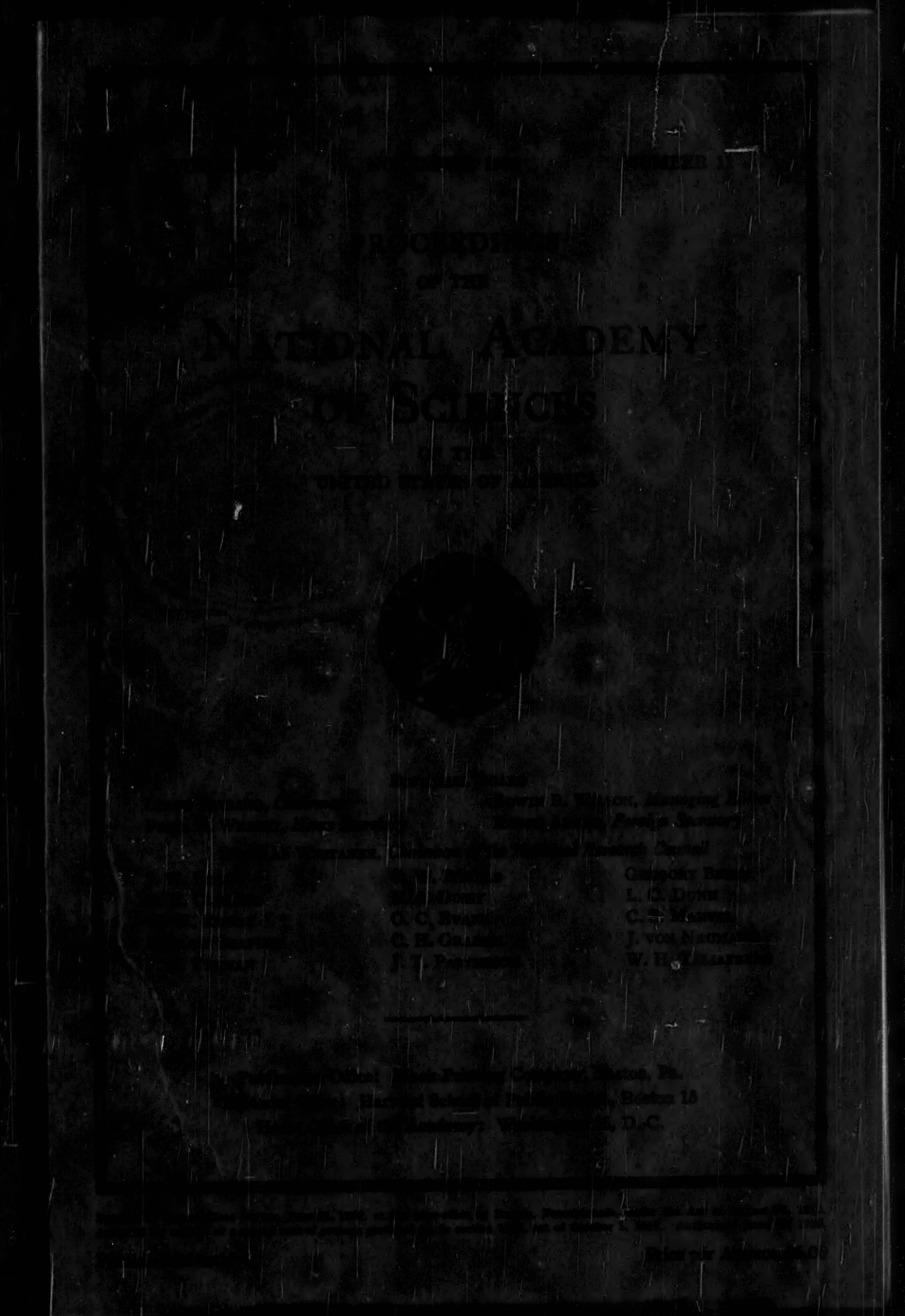

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*ON THE CYTOPLASMIC NATURE OF "LONG-TERM ADAPTATION" IN YEAST**

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Communicated by M. M. Rhoades, September 1, 1950

Introduction.—The ability of substrate to influence the segregation of the capacity to form the corresponding adaptive enzyme system was indicated[1, 2] by experiments designed to investigate this possibility in the case of melibiose fermentation in yeast. In this study a heterozygote was employed which regularly yielded the expected 2:2 ratio of positives to negatives in the absence of substrate. However, when the heterozygote diploid was adapted to melibiose and the cells kept in contact with the substrate during all subsequent procedures, the 4 haplophase segregants yielded a 4:0 ratio of fermenters to non-fermenters in 6 out of 7 instances.

A fundamental assumption of these studies was that the diploid heterozygote involved the mating between positive and negative spores differing in a single gene. It was further assumed that the negatives carried a recessive allele incapable of initiating the formation of the adaptive melibiose system. On the basis of these assumptions and the results obtained in the presence of substrate, it was concluded that, once initiated by the corresponding gene, enzyme formation could continue indefinitely in its absence. Lindegren[3] drew similar, though not identical, conclusions from an analysis of the segregants from a diploid heterozygotic for galactose fermentation.

Winge and Roberts[4] published results on what they call "long-term adaptation" which confirmed the apparent ability of substrate to modify the Mendelian ratios expected in the segregation of a diploid heterozygotic for a single gene. These authors, however, proposed a different explanation and one which they implicitly assume avoids the necessity of invoking transmissible cytoplasmic determinants of enzyme formation.

These authors suggest that their own results, as well as the previously cited melibiose experiments, are consistent with the concept that crosses

between negatives and positives with respect to carbohydrate fermentation in the yeasts do not always involve absolute genetic negatives and positives in the sense of complete absence of functional activity for the former, and presence of full functional activity for the latter. They would assume rather that wherever substrate can modify observed segregation ratios one is dealing with an instance in which a mating is being made between "slow" (the negative strain) and "fast" (the positive strain) genes. On this basis, after sufficient time has elapsed in contact with substrate, it would be impossible to distinguish the two strains since both would exhibit full enzyme activity. The ratios observed would therefore depend on the previous history of contact with substrate.

There is, however, one feature about the phenomenon of long-term adaptation as reported by Winge and Roberts, and confirmed during the course of the present investigation, which is not easily explained by the "slow" gene hypothesis. Once a slow adapting stock has become phenotypically positive in terms of enzyme activity it remains so indefinitely on continued subculture in the presence of the adaptive substrate and can maintain the character for a varying number of cell generations even in the absence of substrate.

One must conclude consequently that two changes have occurred during the 7-day adapting period required to convert the slow strain to a positive. One is, of course, the accumulation of sufficient enzyme to exhibit enzyme activity. The other is that the rate of enzyme formation has been greatly increased and that this augmented capacity for enzyme formation is inherited during subsequent vegetative generations.

In any phenomenon involving the appearance of a heritable modification it would seem necessary to rigorously rule out mutation and selection as the operating mechanism before entertaining other hypotheses. This is particularly essential in connection with long-term adaptation since it is always accompanied by considerable growth of the negative phenotype during the preadaptive period.

The experiments offered by Winge and Roberts on this issue, while highly suggestive, are not adequate for a decisive conclusion. Reversible loss and gain of a populational character under specified alterations of environmental conditions do not preclude the involvement of mutation and selection.[8] Further, the fact that 12 cultures derived from 12 distinct single-cell isolates behaved similarly is likewise not conclusive. By the very nature of the test for phenotype, scoring in fermentation tubes cannot be made until a population density of between 1×10^7 and 1×10^8/cc. is achieved. By this time, under the selective conditions which exist, and with a mutation rate of reasonable frequency, the populations might well be similar in composition quite independently of the nature of the single cell from which they were derived.

It is clearly essential to analyze what occurs within the population during the 6–7 days prior to the appearance of activity. The first question to be answered is whether or not any heterogeneity with respect to enzyme producing capacity exists within the population for unless this is present, mutation and selection cannot be considered as a descriptive mechanism of the phenomenon. If heterogeneity can be established it then becomes pertinent to inquire into its origin.

The present paper presents some of the results of an investigation of the basic mechanism underlying "long-term" adaptation to galactose. We shall confine ourselves here only to those results directly relevant to the origin and transmission of the capacity to form enzyme rapidly.

The results indicate that it is not a mutational phenomenon since a variance analysis performed according to the method of Luria and Delbrück[6] indicates that the heritable modification does not occur in the absence of substrate. Further, reversion from the fast to the slow phenotype on growth in the absence of substrate can be shown to be a mass change, occurring in over 90 out of a hundred cells at the time of reversion.

While the present investigation was in progress, Mundkur and Lindegren[7] published a paper in which they concluded that the phenomenon of long-term adaptation was mutational. They report the data of a variance analysis but do not calculate the variance. Calculation from their data yields a variance of 27.1 with a mean number of "mutants" per sample of 9.6. Assuming their estimated "mutation" frequency of 1×10^{-7} they should have obtained a variance in the neighborhood of 4000, if a mutational process were operating. Thus, if any conclusion is derivable from this experiment it is that "mutation," independent of the presence of galactose, is not involved.

Unfortunately, no conclusion can with certainty be drawn from their published experiment since none of the conditions necessary for the performance of a variance analysis of this kind were satisfied. The absence of a large variance under these conditions signifies nothing.

Methods.—(*A*) *Strain:* The yeast strain C_1d employed in these studies was a representative of the same species used by Winge and Roberts, Saccharomyces Chevalieri. It was derived as a single clone isolate from culture number NRRL-Y1345 supplied through the courtesy of Dr. L. J. Wickerham of the Northern Regional Laboratory. Under comparable circumstances, the behavior of this strain with respect to galactose fermentation was the same as that reported by Winge and Roberts.

(*B*) *Media:* The basic medium was made by adding the following to 1 liter of H_2O; Bacto-peptone, 5 g.; $MgSO_4$, 1 g.; KH_2PO_4, 2 g.; Difco-yeast extract 2.5 g. Agar (20 g.) was added when a solid medium was required. This medium, containing no added carbohydrate, is referred to in the text as B-medium.

Glucose medium contained, in addition to the above, 40 g. of glucose. The galactose medium was made by adding 20 g. of purified galactose to a liter of B-medium. Merck's galactose was purified by recrystallization from 70% alcohol. The use of purified galactose was found necessary for adequate control over the quantitative results obtained since the small amount of glucose normally present as a contaminant in galactose preparations modified the results considerably.

Eosine-Methylene Blue (EMB) test plates were made by adding 5 cc. of 0.5% Methylene Blue and 20 cc. of 2% Eosine to a liter of the galactose agar medium.

(*C*) *Adaptation Times:* Tests for adaptation times were carried out with Durham tubes, a culture being scored as positive when active release of gas into the inverted vial was noted. With this method a "fast" culture appears as positive in 1–2 days, whereas, a "slow" culture takes 5–7 days. Enzyme activity was assayed manometrically with the Warburg apparatus. The rate of anaerobic CO_2 release from galactose was taken as a measure of the activity level of the adaptive enzyme.

(*D*) *Counts:* Direct counts were made with an hemocytometer. Viable and differential counts were made by the use of spread plates. Aliquots ranging from 0.05 to 0.2 cc. were uniformly spread on the surface of the agar with a sterile bent glass rod.

Experimental Results.—(*A*) *Phenotypic Composition of "Slow" Adapting Population:* Analysis of glucose grown cultures of the slow-adapting stock C_1d on EMB-galactose plates quickly revealed the existence of heterogeneity. Two types of clones were apparent after four days of incubation at 30°. The predominant type was small (about 1 mm. in diameter) and possessed the pink color characteristic of a non-fermenter. The other was larger (between 3 and 4 mm. in diameter) and possessed the heavy coloration characteristic of a fermenter. The behavior of these two types in Durham fermentation tubes paralleled their appearance on the test plate. The large pigmented form produced gas in 1–2 days, whereas the small pink clones took from 4–5 days. In subsequent discussions we shall refer to the large pigmented clones as "positive" and to the small pink as "negatives."

Since one is observing *clonal* characteristics on such a test plate, it follows that the differences between the two types must be heritable. This immediately raises the question as to the mechanism underlying the production of these two types.

Before undertaking the description of the experiments pertinent to this question, it is desirable to state briefly certain characteristics of these two clonal varieties. It was generally found that 24-hour glucose grown cultures of C_1d produced one positive clone for about every thousand cells plated. Analysis of the composition of positive clones by restreaking on

EMB-galactose test plates indicated that they were for the most part composed of cells capable of giving rise to positive clones. A similar examination of negative clones revealed that anywhere from 10–60% of their component cells could potentially give rise to positive clones. The presence of negative cells evidently inhibits the expression of the positive phenotype by potentially positive cells. This inhibitory action explains the sharp drop in percentage of positive colonies often observed in very heavily seeded plates, as is, for example, illustrated in the experiments of Mundkur and Lindegren[7] who inoculated as many as 2×10^8 cells in a single plate. The inhibitory effect of negative cells also plays a role in determining the duration of the long-term adaptation process in liquid media.

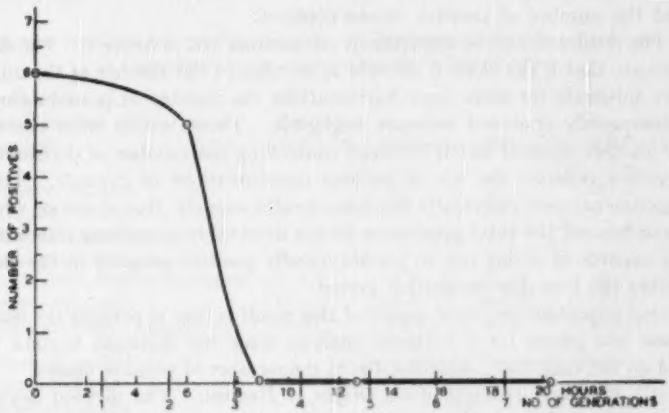

FIGURE 1

The effect of adding galactose at different periods of clonal development on the number of positive clones observed. Each point represents the average of three test plates to which galactose was added at the period indicated.

The ability of the potential positives to ultimately escape from this inhibition is demonstrated by the invariable heavy positive papillation which attends prolonged incubation of the negative clones.

(*B*) *Inhibition of Positive Phenotype by Negative Cells:* It was necessary to determine how early in the development of a clone the presence of phenotypic negative cells exerted their capacity to inhibit the expression of the positive phenotype. To do this, advantage was taken of the fact that an adaptive character was being followed, i.e., one that is not present in a cell prior to exposure to substrate.

The growth rate of 24-hour C_1d glucose-grown cells on solid EMB-B medium was determined by spreading a known amount of cells on the surfaces of a series of plates, which were then incubated at 30°C. for various periods of time. Subsequent to these periods of incubation, the cells were respread with 0.1 cc. of sterile water. The increased number of clones observed provided an estimate of the growth rate. Three separate determinations gave an average value of 0.38 generation per hour. Knowing the growth rate under these conditions made it possible to introduce substrate after a known number of divisions had occurred.

A series of EMB-B plates were seeded with 7000 C_1d glucose-grown cells. These were then incubated at 30°C. for various periods of time, subsequent to which galactose was added in the form of 4 cc. of a 10% solution in 2% agar. The plates were then incubated at 30°C. for 4 days and the number of positive clones counted.

The results of such an experiment are summarized in figure 1. The data indicate that if the clone is allowed to develop in the absence of the adaptive substrate for more than 3 generations the number of positive clones subsequently observed becomes negligible. These results were checked by another method which involved controlling the number of divisions of negative cells by the use of various concentrations of cyanide. These experiments gave essentially the same results, namely, that divisions which occur beyond the third generation do not detectably contribute cells which are capable of giving rise to phenotypically positive progeny in the clone within the four-day incubation period.

One important practical aspect of this result is that it permits the use of these test plates for a variance analysis since the divisions beyond the 3rd do not contribute significantly to the number of positive clones.

(*C*) *Variance Analysis of the Origin of Positives:* The method devised by Luria and Delbrück[6] to decide whether a particular "mutant" type arises randomly or is induced by the testing or selective agent depends on the distributions of mutant types in a series of separate cultures developed in the absence of the agent. The induction hypothesis would predict a distribution yielding a variance characteristic of the usual errors of sampling, i.e., the variance would be of the same order of magnitude as the mean. The mutational hypothesis, on the other hand, presumes that the heritable modification can occur in the absence of the agent and hence at any time in the history of the development of the separate cultures from which the samples are being taken. Accordingly, in sampling from such tubes, one counts not only the mutants but also all the progeny derived from them between the moment of mutation and the time the sample is removed. Under these circumstances, it would be expected that the variance of the distribution of mutants would be much larger than that due to sampling error. Luria and Delbrück[6] derive an expression which

permits one to estimate what they call the "likely" variance on the basis of the random mutation hypothesis.

Several criteria must be satisfied if a variance analysis of this nature is to be meaningful. The medium employed in the development of the separate cultures from which samples are removed must be one that permits equal growth rates of the normal and "mutant" types. The populations must not be allowed to go into the stationary phase where selective forces would be difficult to control and which might result in the elimination or severe suppression of one of the types being followed. The testing conditions which distinguish between the two phenotypes must be such that contributions of the mutant type due to growth of the negative under the testing conditions must not be significant. Finally, the

TABLE 1

VARIANCE ANALYSIS OF THE APPEARANCE OF POSITIVES IN SAMPLES OF SEPARATE CULTURES GROWN IN THE ABSENCE OF GALACTOSE

Exp. number	41	67	67a	95
Growth medium	Glucose	B	B	B
Test plate	EMB-PG	EMB-PG	EMB-PG	EMB-PG + NaCN
Number of cultures	10	10	20	10
Volume of cultures, cc.	1	0.5	0.5	1
Number of organisms/cc.	1×10^8	1×10^8	1×10^8	5×10^8
Volume of samples, cc.	0.2	0.1	0.1	0.4
Mean number of positives/sample	5.0	24.6	21.6	18.9
Observed variance	9.1	35.0	24.8	112.3
Estimated "likely" variance	2000	2000	4000	10,000

Variance of method	Multiple samples from same culture		
Volume of sample	0.2	0.1	0.4
Number of samples	5	10	10
Mean number of positives/sample	2.4	20.4	9.8
Variance	6.2	28.8	43.4

NOTE: All cultures incubated at 30°C. in standing tubes. See text for further details.

inoculum used in seeding the separate tubes must be low enough so as to insure against the probability of seeding a mutant cell initially and, at all costs, the seeding of an equilibrium population of mutant and normal types must be avoided.

It was found that the B medium, as well as the glucose medium, satisfied the first criterion mentioned. The former supported a growth rate equivalent to 0.34 generation per hour and the latter 0.67 generation per hour for both types. In the series of experiments to be described, the populations were sampled when they reached the level of approximately 1×10^8 organisms per cc., which is well below the stationary populations achieved in either of the two sorts of media employed. In view of the results in the

previous sections, it is evident that the EMB galactose plate can be used as a test plate for such analysis. Finally, in all the experiments recorded, the separate cultures were seeded with between 1 and 10 cells each, so that the probability of seeding a mutant was small and the chances of seeding an equilibrium population were nil.

An estimate of the apparent "mutation" frequency was made by the "median" method of Lea and Coulson[8] and yielded an average value of 5×10^{-8} "mutations" per cell per division. Using this figure, it was calculated that the employment of 10 separate cultures, allowed to grow up to a population density of about 1×10^8 per cc. should yield a "likely" variance of 2000 and over if mutations leading to greater capacity for enzyme formation were occurring in the separate cultures without substrate.

Table 1 summarizes a series of variance determinations performed under the conditions noted above. The first three columns present three such determinations which employed the EMB galactose test plates. The last column, Exp. 95, is one of a series of experiments testing the adequacy of the EMB galactose plate by employing a test plate which prevents significant growth of the negative type. A negative cell in contact with the pure galactose medium must depend primarily upon oxidative metabolism for growth. Consequently, the introduction of anaerobiosis of various degrees limits the capacity of such cells to develop. Cells which adapt, however, can continue to grow and give rise to macroscopic clones under such circumstances. The usual anaerobic jars were not found suitable and recourse was had to the use of sodium cyanide. The cyanide was added with the cell suspensions as they were spread and the plates were then sealed individually with either plasticine or Scotch tape. This sealing was essential in order to retain the cyanide during the incubation. It was empirically established that 0.5 cc. of 10^{-1} M sodium cyanide added in this manner was sufficient to prevent significant growth of the negatives and yet permit the adaptation and growth of the positives.

It is evident from the data that the observed variance in the number of positives is not very much different from the mean and certainly very much below that expected from the mutational hypothesis. The variance of the method, determined from a number of samples from the same tube, is included in the lower portion of table 1. These are of the same order of magnitude as the experimental variances observed from the different cultures. It will be noted that the variance of the cyanide method is higher than that obtained with the plain EMB galactose plates. This is a reflection of the difficulty of maintaining identical concentrations of cyanide in the separately sealed plates during the incubation period. Such variation from plate to plate increases the variance above that to be expected from the error of sampling. This higher variance is also of course seen in the experimental samples derived from the separate cultures.

The experimental variance is nevertheless proportionately as far below the "likely" variance as the others and furthermore agrees with the variance of the cyanide method itself.

It seems necessary to conclude from these data that "mutations" to the positive phenotype do not occur in cultures developed in the absence of

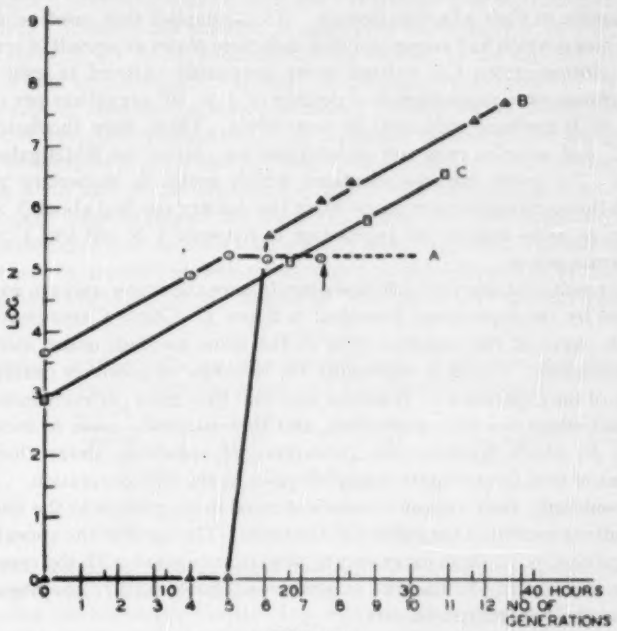

FIGURE 2

The reversion of positive cells to the negative phenotype during growth in the absence of substrate. Curve *A* represents the number of positives, and curve *B* the number of negatives during the reversion. Curve *C* represents the growth of negative cells alone in the same medium under the same conditions. The arrow indicates the point at which a transfer was made to a fresh tube in the reversion experiment.

substrate. Thus, the heritable modification leading to rapid enzyme production which occurs in certain cells during long-term adaptation is induced by the adaptive substrate.

(*D*) *Reversions of Positives to Negatives:* The next question requiring analysis was the mechanism underlying the reversion of "fast" cultures to the original "slow" type, during growth in the absence of substrate. Here

again, use was made of the EMB-galactose test plate to examine the details of the conversion.

The irregularities in the times required for reversion as recorded by Winge and Roberts[4] suggested the desirability of studying this phenomenon under relatively well-controlled conditions. In particular, it seemed preferable to begin the examination with positive clones which were comparable in their adaptive history. To accomplish this, positive clones were chosen which had arisen on EMB-galactose plates as a result of spreading a glucose-grown C_3d culture never previously exposed to galactose. Such clones were suspended to a density of 1×10^4 organisms per cc. in 5 cc. of B-medium contained in test tubes. These were incubated at 30° C. and samples removed at intervals for plating on EMB-galactose plates. To avoid the complications which ensue in stationary phase populations, transfers were made when the density reached about 5×10^8 per cc. so as to restore the population to between 1×10^4 and 1×10^5 organisms per cc.

The results obtained in such experiments were extremely uniform and are typified by the experiment described in figure 2. Curve *C* represents the growth curve of the negative type in the same medium under identical circumstances. Curve *A* represents the behavior of positives during the course of the experiment. It will be seen that they grow perfectly normally up until about the fifth generation, and then suddenly cease to increase. Curve *B*, which describes the appearance of negatives, shows that the number of negatives is quite negligible prior to the fifth generation. Then quite suddenly, they appear in numbers equal in magnitude to the number of positives present in the culture at the time. The burst in the appearance of negatives, is, in these experiments, always coincident with the cessation of the increase in the number of positives. Subsequently the negatives grow at their characteristic rate.

It would appear that the positive cells are capable of producing positive buds for about five generations. Quite suddenly, however, the vast majority of the positive cells begin to produce negative buds. Despite the fact that they are producing negative buds it is apparent from the constancy in number of positives that they are still phenotypically positive in the sense that they can give rise to positive clones when brought into contact with substrate on a test plate. It was not possible in this particular experiment to determine the ultimate fate of these positives. Other experiments indicate that they do not remain as such indefinitely. It has proved experimentally difficult to decide, however, whether their final disappearance is due to death or conversion to negatives.

The possibility that the burst of negatives observed in this and other similar experiments may be due to a marked, but transient, stimulatory effect of the presence of positives on the growth of negatives can be ruled

out on several grounds. In the first place, the apparent stimulation leads to growth rates too high to be reasonably attained, being, in some experiments, 20 times the normal growth rate of the negative type. Further, reconstruction experiments in which the growth of negatives was followed in artificial mixtures of positives in varying proportions exhibited no such stimulation prior to the 5th generation, the normal time of reversion of the positives. Finally, the number of negatives which appear in any given experiment at the burst is quantitatively related to the number and the growth rate of the positives present at that particular moment. This is illustrated by the data presented in table 2 which presents results of representative reversion experiments. In this table are calculated the growth rates in terms of generations per hour of the positives in the period prior to the burst and of the negatives in the period beyond the burst. The "apparent" growth rate of the negatives during the burst is calculated on the assumption that negatives derive only from negatives. It will be seen that these "apparent" growth rates range anywhere from 6 to 16 times the normal growth rates of the negatives. A "corrected" growth rate was

TABLE 2

COMPARISON OF GROWTH RATES (GENERATIONS/HOUR) OF POSITIVES AND NEGATIVES DURING VARIOUS PERIODS OF A REVERSION EXPERIMENT

EXP. NO.	POSITIVE IN PRE-BURST PERIOD	NEGATIVE IN POST-BURST PERIOD	POSITIVE AT BURST	NEGATIVE AT BURST	
				APPARENT	CORRECTED
118	0.37	0.36	0.01	4.73	0.39
121a	0.36	0.36	<0	2.13	0.35
121b*	0.17	0.19	0.001	1.41	0.16
127	0.38	0.37	0.003	5.99	0.36

* NOTE: "Apparent" growth rate at burst was calculated with the assumption that negatives are derived from negatives. "Corrected" growth rate determined on the assumption that negatives are derived from negatives and positives. All experiments except 121b were performed at 30°C. The latter was carried out at 14°C.

calculated on the assumption that the negatives during the burst period are derived both from the small number of negatives present as well as from the positives. It is seen that the growth rates thus obtained are, within experimental error, identical to the growth rates of the positives and the negatives during periods when each is derived from its own type.

The good agreement among the various growth rates indicates that the production of negative progeny by the positive cells is proceeding at the normal division rate of these cells and that the vast majority of the positive cells present participate in the production of negative buds.

Experiment 121b, which was performed at 17°C. rather than 30°C., offers further convincing evidence for these conclusions. At this temperature, the growth rates of the positives and negatives are less and the corrected growth rate of the negatives during this period corresponds to the growth

of the positives at this lower temperature. It is noteworthy that the number of generations required for the burst is not significantly different at the slower growth rate, although the time of its occurrence is of course about doubled.

So long as attention is confined to recently derived positives, the results obtained in reversion experiments are quite uniform. The reversions are massive ones involving most of the cells present and generally occur between the 5th and 6th generations. The number of generations required for the reversions has been, within the range studied (0.20 to 0.67 generations/hr.), relatively independent of the division rate.

When, however, the reversion phenomenon is studied with clones derived from cultures subjected to prolonged growth in the presence of galactose, great variability is observed both in the number of generations required for the burst and in the percentage of individuals participating when it does occur. The irregularity exists not only among cultures undergoing independent serial passage in galactose but also among the individual cells of a given culture. Thus, one clone from a culture may not revert even after 70 generations whereas another may show evidence of reversion after 6 generations. Little can as yet be said about the nature of this irregularity except that it is evidence for some discontinuous process affecting the ability to preserve the capacity of rapid enzyme formation during growth in the absence of substrate. In general it may be noted that serial subculture in the presence of substrate increases the number of individuals requiring a large number of generations before reversion occurs. It is this phenomenon which is undoubtedly the basis for the irregularity observed by Winge and Roberts[4] in their reversion experiments.

Discussion.—(*A*) *Nature of the Phenomenon at the Populational Level:* Winge and Roberts[4] explain the long delay preceding the appearance of enzyme activity by assuming that they are dealing with cells which form enzyme slowly at a rate determined by the possession of a slow gene. Aside from the fact that it fails to provide an explanation of the heritable nature of the adapted state it is evident that the above hypothesis is inconsistent with the data obtained in the present analysis.

Account must be taken of the demonstrated heterogeneity in the original population. Under the experimental conditions described, there are two factors which appear to determine the duration of the adaptation times in such slow strains.

(1) Only a small proportion of the individuals seeded are actually capable of undergoing the adaptation.

(2) The presence of phenotypic negatives inhibits the complete expression of the positive phenotype.

A simple calculation makes it evident that the second factor is a quantitatively important one. From the viewpoint of the first factor, the

difference between seeding a thousand cells of a "slow" stock and a thousand cells of a "fast" stock is that, in the former, one is seeding only one adaptable cell and in the latter a thousand. With a generation time of about 0.67 generation per hour this thousandfold difference in initial inoculum should be made up in 10 generations or about 16 hours. It is clear that this alone does not explain the 4–5 day difference in the adaptation times between fast and slow stocks. Consequently the inhibitory mechanism forms a quantitatively important component of the phenomenon of long-term adaptation.

It should further be noted that the actual speed of adaptation of those cells in a "slow" population which do adapt compares favorably with the rate of adaptation observed with cells from fast stocks. The experiments cited indicate that adaptation occurs within 3 generations, i.e., 10 hours or less. This compares with 5 hours required for full adaptation in cells from fast stocks.

(*B*) *The Nature of the Phenomenon at the Cellular Level:* The evidence presented indicates the involvement of a heritable modification in rate of enzyme production induced by the presence of substrate. The mass reversion which occurs on growth in the absence of substrate makes it highly unlikely that a substrate-directed genic change is involved, and in any case, removes the criterion of genic stability necessary for testing this possibility by classical genetic methods.

Under these circumstances it seems difficult to avoid looking for a cytoplasmic basis to explain the heritable nature of the change. All the data thus far obtained on the phenomenon can be adequately explained by the existence of cytoplasmic units possessing the following properties: (1) they are concerned with enzyme formation; (2) their number increases more readily in the presence of substrate; (3) their law of growth is autocatalytic.

Previous studies[9–11] of enzymatic adaptation in resting cells of "fast" strains have revealed that the kinetics of enzyme formation are autocatalytic in nature and that the enzyme forming systems interact in a competitive manner. These and other results were summarized[9, 10] in a concept of gene control over enzyme synthesis which assumed that (*a*) cytoplasmic units (plasmagenes) exist which mediate the formation of specific enzymes, (*b*) substrate stabilizes the enzyme-forming complex and thus leads to an increased net rate of formation of both enzyme and cytoplasmic unit; and finally (*c*) the law of growth of these elements is autocatalytic.

It is evident that the conclusions derivable from the investigations of both normal and "long-term" adaptation are similar. The latter phenomenon, involving as it does transmission during cell generation, represents more decisive evidence for the autocatalytic growth of the cytoplasmic

determinants. In addition, the discreteness exhibited in both the appearance and loss of the ability to transmit enzyme forming capacity revealed the particulate nature of the underlying mechanism with a clarity not possible with the fast adapting strains.

In this connection it should be noted that the dramatic suddenness with which the production of negative progeny ensues finds ready interpretation in the mode of cell production characteristic of the yeasts. The fact that a yeast bud receives only 10% of the parent cytoplasm has two important numerical consequences for cytoplasmic transmission. One is that the probability of the daughter cell obtaining a particular cytoplasmic element is 1:10 rather than 1:2 for the usual mechanism of cell division. The second is that each cell generation results in a 1:10 dilution of parental cytoplasm. Thus, by the 5th generation in a yeast clone, a 1:100,000 dilution has occurred as compared with a 1:32 dilution in a form such as paramecium.

The crux of the problem has always been, and still is, the nature of the particulate elements involved and the degree of autonomy to be ascribed to them by virtue of their behavior and autocatalytic growth.

Perhaps the simplest explanation of the autocatalytic behavior is one which depends upon the fact that the enzyme being followed is one which is involved in the energy generating mechanism. Thus the more enzyme present, the greater the possible rate of further enzyme synthesis.[10, 11] This suggestion has been made untenable by experiments with C_1d positives which will be detailed in a separate publication. It was possible to demonstrate that cells in which none of the adaptive galactozymase could be detected still retained the capacity to form enzyme rapidly and furthermore could transmit this ability to their progeny. It appears that particles other than the active enzyme molecules themselves are responsible for the ability to form more replicas.

The existence of a genic background to the phenomenon in terms of a single gene difference between the "fast" and "slow" varieties has been well established[4] and constitutes the only important difference from the studies of Sonneborn[12] and his collaborators on the inheritance of antigenic type in paramecia. The reversion experiments exhibiting the production of progeny incapable of forming an enzyme which the parent cell can synthesize are very similar to the phenomenon reported by Ephrussi[13] and his coworkers on the inheritance of the ability to form cytochrome-oxidase and succinic dehydrogenase in yeast. It should however be noted that in the latter instance the loss is permanent. In the case of the adaptation to galactose it is not. A certain small proportion of the progeny derived from a negative cell regain the capacity on being brought into contact with substrate and hence a replenishing mechanism must be presumed to exist.

Whatever the detailed mechanisms turn out to be, it seems likely that the phenomenon of long-term adaptation can provide an analytical tool which could help answer some of the questions raised by the previous studies on enzymatic adaptation. In principle it could provide information not easily attainable in any other way, for with its aid a particle analysis analogous to the admirable studies of Preer[14] and Sonneborn[15] in paramecia may now be feasible in the case of enzymatic adaptation in the yeasts.

Summary.—The phenomenon of "long-term adaptation" to galactose fermentation by yeast has been analyzed. The possibility that it is based on mutation and selection has been eliminated by a variance analysis and the demonstration that reversion to the original type is a mass phenomenon.

The data indicate that contact with substrate induces, in a small proportion of cells, a modification leading to ability to form enzyme. This modification is transmitted from one cell generation to the next by cytoplasmic elements which increase autocatalytically. These conclusions concerning the cytoplasmic factors determining enzyme formation are in agreement with those deduced from previous studies of enzymatic adaptation in fast adapting strains.

The importance of the phenomenon and its experimental analysis stems from the ability to thus exhibit the discrete nature of the cytoplasmic components determining enzyme synthesis. A more adequate quantitative approach to the nature of these units is thus made possible.

* This investigation was aided by a grant from the National Cancer Institute of the U. S. Public Health Service.

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[2] Lindegren (*Cold Spring Harbor Symp. Quant. Biol.*, 11, 115–129 (1946)) reported difficulty in repeating the melibiose experiments. Subsequent publication in 1949 (*The Yeast Cell*, Educational Publishers, St. Louis, 1949, Chapter 26, p. 19) of the data on which these doubts were raised makes it possible to examine their relevancy to the earlier experiments. Several features make interpretation of these later experiments difficult and comparison with the previous results impossible. The cross employed in the original experiments consistently gave the normal 2:2 segregations of +:− in control matings. The "control" cross used by Lindegren for the repetition yielded only 1 normal 2:2 out of 9 complete tetrads analyzed. Five of the asci yielded a 1:3 ratio of positive to negatives and the remaining 2 asci did not possess the positive allele in any of the 4 spores! Furthermore, the plus and minus strains used in the experimental matings in the presence of substrate were not the same as those used in the experimental cross. It may well be questioned if these two sets of matings are at all comparable. Finally, if they are considered to be comparable, it seems necessary to entertain the probability that the presence of substrate had an effect on the segregation of positives. As compared with 1 out of 9 in the controls, all four complete tetrads analyzed in the experimental series yielded a 2:2 ratio. The probability of this being due to chance is in the neighborhood of 1 in 10^4.

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NON-ENZYMATIC OXIDATION OF TYROSINE AND DOPA

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Communicated by L. C. Dunn, August 3, 1950

Introduction.—In the course of studies demonstrating the existence of tyrosinase activity in normal pigmented mouse skin, to be reported elsewhere, it was noted that under certain conditions not only dopa (3,4-dihydroxyphenylalanine) but also tyrosine was capable of undergoing non-enzymatic oxidation. The necessary conditions for the non-enzymatic oxidation of tyrosine involved the presence of both cupric ions and a "threshold concentration" of dopa. It was also observed that the oxidation of tyrosine ended even though most of the tyrosine remained in the system unaffected.

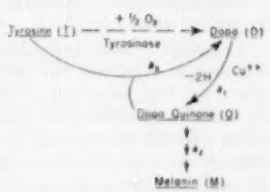

FIGURE 1

Simplified scheme for the non-enzymatic oxidation of tyrosine and dopa in the sequence leading to the formation of melanin.

The mechanism proposed to account for the non-enzymatic oxidation of tyrosine involves the role of dopa quinone as an electron and proton acceptor, of tyrosine as an electron donor and of cupric ion as an electron transporter. The over-all chemical reaction can then be written

$$\text{Tyrosine} + \text{Dopa quinone} + H_2O = 2\ \text{Dopa}.$$

The reaction sequence is then readily visualized as in the simplified scheme

shown in figure 1. On the basis of this scheme it is possible to perform a kinetic analysis with consequences that can be tested experimentally.

Kinetic Analysis.—From the system shown in figure 1 it is possible to set up the following rate equations (assuming that the reactions are first order with respect to the various reactants):

$$\frac{dQ}{dt} = -k_2Q - k_3QT + k_1D, \tag{1}$$

$$\frac{dD}{dt} = k_3QT - k_1D, \tag{2}$$

$$\frac{dT}{dt} = -k_3QT. \tag{3}$$

Equation (3) is readily solved for T to give

$$T = c_1e^{-k_3\int Q\,dt}. \tag{3a}$$

Substitution for T from equation (3a) in equation (2) gives

$$\frac{dD}{dt} = k_3Qc_1e^{-k_3\int Q\,dt} - k_1D. \tag{2a}$$

Integration of this linear equation gives

$$D = Ce^{-k_1t} + k_3c_1e^{-k_1t}\int Qe^{-k_3\int Q\,dt}e^{k_1t}\,dt. \tag{4}$$

Substituting the value for k_3QT from equation (3) in equation (2), we have

$$\frac{dD}{dt} + k_1D = -\frac{dT}{dt}. \tag{2b}$$

On integrating this linear equation, we have

$$D = T_0e^{-k_1t} - T + k_1e^{-k_1t}\int Te^{k_1t}\,dt. \tag{5}$$

Now, substituting for T from equation (3a) and for D from equation (5) in equation (1), we have

$$\frac{dQ}{dt} = -k_2Q - k_3Qc_1e^{-k_3\int Q\,dt} + k_1[T_0e^{-k_1t} - c_1e^{-k_3\int Q\,dt} + k_1e^{-k_1t}\int c_1e^{-k_3\int Q\,dt}e^{k_1t}\,dt]. \tag{6}$$

It is possible to solve for the variables Q, D and T as functions of t, although with great difficulty.

Since, in the non-enzymatic experiments, the oxidation of dopa and tyrosine ends with most of the original tyrosine still present, a simpler approximate solution of the equations for Q and D may be obtained by assuming that the concentration of tyrosine remains constant. Thus the original equations become

$$\frac{dQ}{dt} = -k_2Q + k_1D \qquad (1')$$

and

$$\frac{dD}{dt} = -k_1D, \qquad (2')$$

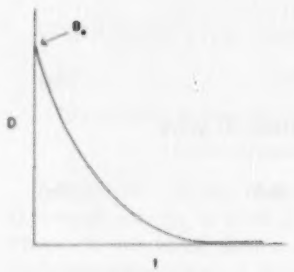

FIGURE 2

Graph of D as a function of t; equation (2'a).

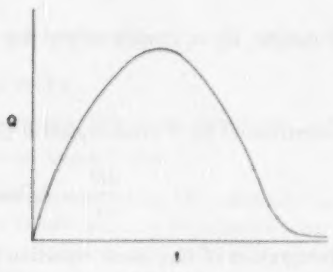

FIGURE 3

Graph of Q as a function of t, with coordinates for maximum value of Q given in equation (5'a) and equation (4'a). Here equation (3') is plotted.

since, from equation (3), $\frac{dT}{dt} = -k_3QT = 0$.

Solving for D in equation (2') gives

$$D = D_0e^{-k_1t}. \qquad (2'a)$$

This equation, when plotted, gives the familiar first-order curve shown in figure 2.

Substituting the value for D from equation (2'a) in equation (1'), we have

$$\frac{dQ}{dt} = -k_2Q + k_1D_0e^{-k_1t}. \qquad (1'a)$$

On integrating this linear equation, and knowing that $Q = 0$ at $t = 0$, we have

$$Q = D_0\frac{k_1}{k_1 - k_2}(e^{-k_2t} - e^{-k_1t}). \qquad (3')$$

The following deductions can be made from equation (3'):

1. Since Q cannot have negative values, $k_1 > k_2$.
2. $Q = 0$ at $t = 0$, and $\lim_{t \to \infty} Q = 0$.
3. Since $Q = 0$ at zero and at infinite times, it must have a maximum value.

Differentiating equation (3'), setting the differential expression equal to zero and solving for t at the maximum for Q results in the following:

$$t_{max.} = \frac{\ln (k_1/k_2)}{k_1 - k_2}. \qquad (4')$$

If $k_1 \gg k_2$ (as will be justified later), then

$$t_{max.} = \frac{\ln (k_1/k_2)}{k_1}. \qquad (4'a)$$

Now, substituting value for $t_{max.}$ from equation (4') in equation (3') and simplifying, we have

$$Q_{max.} = D_0\frac{k_1}{k_1 - k_2}\left(\frac{k_1}{k_2}\right)^{k_1/(k_2 - k_1)}\left(\frac{k_1 - k_2}{k_2}\right). \qquad (5')$$

If $k_1 \gg k_2$, then

$$Q_{max.} = D_0. \qquad (5'a)$$

Equation (3') is plotted in figure 3.

Further important conclusions, which can be checked experimentally, can result from an additional analysis if we make one assumption. This assumption is that the rate of melanin production is proportional to O_2 consumption as measured in a Warburg respirometer. This is justifiable since extraneous interfering or inhibitory substances are not present in the non-enzymatic system. Since

$$\frac{dM}{dt} = k_2 Q, \; M = k_2 \int_0^t Q \, dt$$

Substituting value for Q from equation (3'), we have, on integration,

$$M = \frac{D_0 k_1 k_2}{k_1 - k_2} \left[\left(\frac{k_1 - k_2}{k_1 k_2} \right) - \left(\frac{1}{k_2} e^{-k_2 t} - \frac{1}{k_1} e^{-k_1 t} \right) \right]. \qquad (6')$$

Expanding the term e^{-kt} into a Maclaurin's series, and using the first three terms, equation (6') becomes

$$M = D_0 k_1 k_2 \frac{t^2}{2}. \qquad (7')$$

It should be noted that equation (7') is arrived at on the assumption that k_1 and k_2 are of the same order of magnitude.

If, however, $k_1 \gg k_2$, then equation (6') can be written, on simplification,

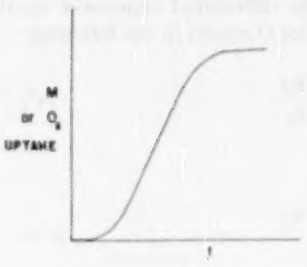

FIGURE 4

Graph of M or O_2 consumption as a function of t; equation (7').

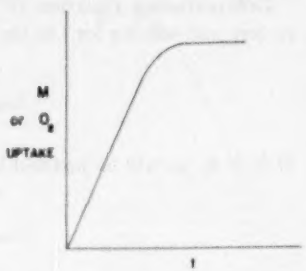

FIGURE 5

Graph of M or O_2 consumption as a function of t; equation (9').

$$M = D_0(1 - e^{-k_2 t}). \qquad (8')$$

Expanding the term $e^{-k_2 t}$ into a Maclaurin's series, and, using the first two terms, equation (8') becomes, finally,

$$M = D_0 k_2 t. \qquad (9')$$

Now, if equation (7') holds, i.e., if k_1 is not much greater than k_2, then, if M or O_2 consumption is plotted against t, the curve should show an induction period for small values of t, as in figure 4. If, however, equation (9') holds, i.e., if $k_1 \gg k_2$, then, if M or O_2 uptake is plotted against t, the curve should be a straight line for small values of t, as in figure 5. Now, actual measurements of O_2 uptake in Warburg respirometers result in the curve shown in figure 5, in the case of the non-enzymatic system.

Thus, the experimental evidence supports the conclusion that $k_1 \gg k_2$.

Discussion.—(A) Mechanisms other than the non-enzymatic oxidation of tyrosine to dopa proposed here have been considered and tested. For example, O_2 consumption in excess of the amount needed for complete oxidation of dopa can be attributed to the formation of hydrogen peroxide in the oxidation of dopa to the quinone stage. Furthermore, the oxidation of tyrosine might be an oxidative deamination, rather than an oxidation to dopa, with consequent increased O_2 consumption. The experimental evidence (to be reported elsewhere), however, is strongly against these other possibilities as explanations for *both* the excess O_2 consumption and correspondingly increased pigment formation observed.

(B) According to the scheme in figure 1, the reaction should not end until all the tyrosine is oxidized. The fact remains, however, that the reaction apparently ends despite the presence of a large amount of unoxidized tyrosine. This is explained by assuming that $k_1 \gg k_2 \gg k_3$, i.e., the rate of dopa formation is much less than the rate of dopa and quinone utilization in melanin formation. Thus the concentrations of dopa and of dopa quinone fall rapidly to a level at which the rate of dopa formation becomes immeasurably slow, and soon thereafter the reaction ends, for all practical purposes. Thus, the operational term "threshold concentration" actually means that concentration of catalyst sufficient to give a measurable reaction.

(C) Aside from the kinetic analysis previously presented, there are several other points of interest arising from a consideration of the non-enzymatic system in which both tyrosine and dopa undergo oxidation:

1. It is a system in which a side reaction has the features of an autocatalytic reaction.

2. It provides (since tyrosinase is a copper enzyme) another example of cases reported by Smith and Lumry,[1] where reactions catalyzed by metal enzymes can be catalyzed by the specific metal ions alone, although in the instance reported here, the metal ion catalysis is far less effective and lacks the specificity of the enzymatic catalysis.

3. It may reflect some conditions in the more complicated enzyme system. Then tyrosinase may have a function in addition to its monophenolase and diphenolase activities; that is to say, tyrosinase may also act as an electron transporter between tyrosine and dopa quinone.

Acknowledgments.—Grateful acknowledgments are made to Amos Norman of the Subcommittee on Biophysics (of the Joint Committee on Graduate Instruction) of Columbia University and to Dr. Alan J. Hoffman of the Mathematics Department of Columbia University for their valuable suggestions in the carrying out of the mathematical analysis.

[1] Smith, E. L., and Lumry, R., *Cold Spring Harbor Symp. Quant. Biol., New York*, 14, 168–178 (1949).

COULOMETRIC TITRATIONS WITH EXTERNALLY GENERATED REAGENTS

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The feasibility of several types of coulometric titrations in which the titrant is generated in the solution containing the sample has already been

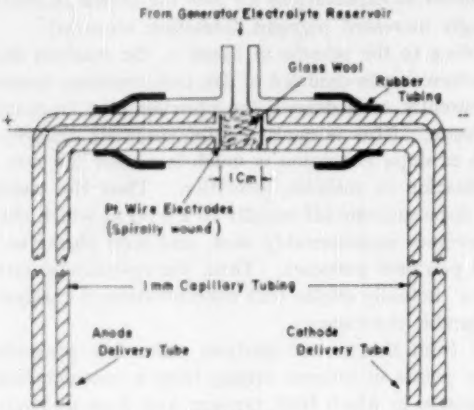

FIGURE 1

Generator Cell

demonstrated.[1, 2] We have found that satisfactory coulometric titrations may also be performed by means of reagents which are electrolytically generated in a separate generator cell and subsequently delivered to the titration vessel.

SAMPLE	NO. OF DETNS.	MEQ. TAKEN	MEQ. FOUND	AVERAGE PRECISION, %	AVERAGE ERROR, %
HCl	4	1.208	1.211	0.1	+0.2
	3	2.417	2.429	0.1	+0.5
NaOH	5	0.841	0.843	0.2	+0.2
	3	1.682	1.689	0.2	+0.4
KHP[a]	4	1.266	1.272	0.1	+0.5
As_2O_3	4	1.152	1.154	0.1	+0.2
	4	2.304	2.307	0.1	+0.1

[a] Potassium acid phthalate.

A simple generator cell which has been employed satisfactorily for this purpose is shown in figure 1. A suitable generator electrolyte was allowed

to flow through the cell constantly during the course of the titration. The effluent solution from the appropriate delivery tube was then allowed to flow into the solution containing the sample.

Typical results which have been obtained with this technique are summarized in the table. The generator electrolyte which, on electrolysis, furnished the hydrogen and hydroxyl ions for the acid-base titrations was 1.0 *M* sodium sulfate and that which furnished the iodine for the arsenious oxide titration was 1.0 *M* potassium iodide. A constant generation current of 0.2500 amp. was maintained in all titrations. The reproducibility of the analyses is excellent. The finite time which is required for mixing apparently causes a significant delay in indicator response at the equivalence point. This delay is believed to be the primary source of the small but consistent positive error which was observed in all determinations. Automatic devices which will anticipate the approach of the end-point and thus permit greater accuracy in the determination of the generation time are now being studied in the hope of eliminating this systematic error.

Further studies on the applicability of this technique to a wide variety of titrations, including precipitation titrations, are now in progress in this Laboratory.

[1] Farrington, P. S., and Swift, E. H., *Anal. Chem.*, 22, 889 (1950). References to previous work by Swift and his coworkers are given in this paper.

[2] Cooke, W. D., and Furman, N. H., *Ibid.*, 22, 896 (1950).

*A NEW INHERITED ABNORMALITY OF HUMAN HEMOGLOBIN**

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Communicated by Linus Pauling, August 31, 1950

In the course of a study of the inheritance of the sickling phenomenon[1] two families were encountered in which there occurred one or more children with a hematological picture which resembled that of sickle cell disease but was of less severity. The situation further differed from that usually encountered in sickle cell disease in another important respect. It has been shown that in the great majority of instances both parents of a child with

sickle cell disease exhibit the sickle cell trait.[1, 2] In each of these two families, however, the erythrocytes of only one parent could be induced to sickle.

Electrophoretic studies[3, 4] have demonstrated the presence of an inherited abnormality of hemoglobin in sickle cell disease and sickle cell trait. Similar studies of various members of these two anomalous families have now been carried out and have led to the recognition of a new inherited abnormality of hemoglobin. The detailed results of the electrophoretic analyses are given in table 1. In family A the hemoglobin of the propositus was found to consist of three electrophoretic components.

TABLE 1

	AGE	SICKLING TEST	PATTERN (FIG. 2)	HEMOGLOBIN COMPONENT,[a] % NORMAL	SICKLE	NEW COMP.
Controls						
Normal	..	−	*a*	100	−	−
Sickle cell anemia	..	+	*b*	−	100[b]	−
Sickle cell trait	..	+	*c*	55–76	24–45[b]	−
Family A						
Father (P. C., Sr.)	29	−	*d*	64.7	−	35.3
Mother (E. M. C.)	28	+	*c*	66.5	33.5	−
Brother (P. C., Jr.)	6	−	*d*	66.4	−	33.6
Brother (R. G. C.)	4	−	*a*	100	−	−
Propositus 1, ♀ (P. A. C.)	3	+	*f*	13	39	48
Family B						
Father (J. W.)	33	−	*d*	69.8	−	30.5
Mother (D. F. W.)	31	+	*c*	68.9	31.1	−
Propositus 2, ♀ (R. W.)	12	+	*e*	−	47	53
Propositus 3, ♂ (T. W.)	10	+	*e*	−	50	50
Brother	8	−	*a*	100	−	−

[a] The authors are indebted to Dr. Ibert C. Wells for carrying out these computations.
[b] Wells, I. C., and Itano, H. A., in press. In some cases the hemoglobin of individuals with the clinical diagnosis of sickle cell anemia contains a small fraction (5 to 20 per cent) of normal hemoglobin.

The mobilities of two of the components correspond to those of the hemoglobins from normal and sickle cell anemic individuals, respectively. The other component, which migrates as a more positive ion than either normal or sickle cell anemia hemoglobin, has hitherto not been encountered. In family B the hemoglobin of each of the two propositi was found to be a mixture of two types, namely, that characteristic of sickle cell disease and the new fraction. Electrophoretic studies of the remaining members of the families revealed that in each family the hemoglobin of the parent whose erythrocytes sickled exhibited the electrophoretic findings typical of sickle cell trait,[4, 5] while the hemoglobin of the other (non-sickling) parent was a mixture of two types, normal and the new component detected in the pro-

positi. The families involved in the study are pictured in figure 1. The propositi through whom the study was initiated are indicated by arrows.

Carbonmonoxyhemoglobin solutions were prepared and analyzed electrophoretically in the Tiselius apparatus; exact details of the preparation and analysis have recently been reported.[5] The carbonmonoxyhemoglobin solutions were diluted to 1.0 per cent concentration and dialyzed against cacodylate-sodium chloride buffer[6] of ionic strength 0.1 and pH 6.50 for eighteen hours. The final scanning diagrams were taken after fifteen hours of electrophoresis at 1.5°C. at a potential gradient of 3.49 to 3.60 volts per centimeter. The mobilities in the ascending boundaries of the carbonmonoxyhemoglobins from normal and sickle cell anemic individuals

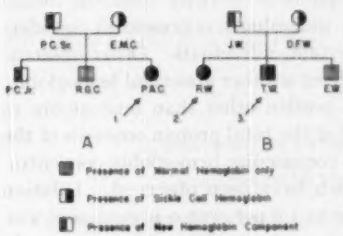

FIGURE 1

The two families under study.

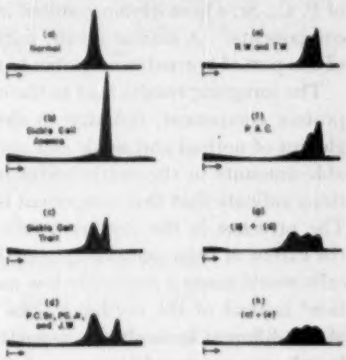

FIGURE 2

The Longsworth scanning diagrams of the carbonmonoxyhemoglobins of the individuals under study compared to the scanning diagrams of the carbonmonoxyhemoglobins of individuals known to be hematologically normal or to have sickle cell anemia or sickle cell trait.

under these conditions are 2.4×10^{-5} and 2.9×10^{-5} cm./sec. per volt/cm., respectively. The mobility of the new component is 3.2×10^{-5} cm./sec. per volt/cm.

The Longsworth scanning diagrams of the ascending boundaries are shown in figure 2. The various components are visible in the scanning diagrams of the descending boundaries, but the resolution of the peaks is poor.[5] Patterns (*a*) and (*b*) are those of the hemoglobins from normal and sickle cell anemic individuals, respectively, obtained under the experimental conditions described above. The hemoglobins of two of the siblings (R. G. C. and E. W.) of the propositi showed the normal pattern. Pattern (*c*), a typical sickle trait pattern containing both the normal and sickle cell

anemia hemoglobins, is similar to those of the hemoglobins of the sickling parents (E. M. C. and D. F. W.) in this study. Pattern (*d*) shows two components, normal hemoglobin and the new component, but lacks the sickle cell anemia component. This pattern was found in both the non-sickling parents (P. C., Sr., and J. W.) and in a brother (P. C., Jr.) of one of the propositi. The hemoglobins of two of the propositi (R. W. and T. W.) gave the two-component pattern, (*e*), which appears to contain sickle cell anemia hemoglobin and the new component but not normal hemoglobin. The hemoglobin of the third propositus (P. A. C.) resolved into all three components, as shown in pattern (*f*). The analysis of a solution prepared by the addition of one part of sickle cell anemia hemoglobin to two parts of P. C., Sr.'s hemoglobin resulted in pattern (*g*), which clearly shows three components. A similar result, pattern (*h*), was obtained by the addition of one part of normal hemoglobin to two parts of R. W.'s hemoglobin.

The foregoing results lead to the conclusion that a previously unreported protein component, differing in electrophoretic mobility from the hemoglobins of normal and sickle cell anemic individuals, is present in considerable amounts in the erythrocytes of certain individuals. Other observations indicate that this component is indeed another abnormal hemoglobin. The presence in the erythrocytes of a protein other than hemoglobin to the extent of from one-third to one-half of the total protein contents of the cells would cause a markedly low mean corpuscular hemoglobin concentration[4] instead of the normal values which have been observed. Dilution of the different hemoglobin preparations to 1.0 per cent concentration was based on a spectrophotometric determination using a standard curve obtained at 540 mμ with normal hemoglobin.[5] Subsequent electrophoretic analyses of these diluted solutions revealed that the total area of the scanning diagram was in each case equal to that obtained with a 1.0 per cent solution of normal hemoglobin. This indicates that within the error of this method the molecular extinction coefficient at 540 mμ is the same for all three components, assuming equal molecular weights. It may be noted that the color of the fast moving component in the ascending boundary was observed to be the same as that of normal carbonmonoxyhemoglobin.

The data which have been presented suggest that the tendency to form the new hemoglobin component is inherited as if due to a single dominant gene. The effect of this gene in the homozygous condition is not yet known; it is possible that it corresponds to some already recognized hematological syndrome. The relationship of this gene to the sickle cell gene is not clear at the present time. The hematological picture in the individuals who may be postulated to have received both genes is explicable either on the basis of the factor interaction on the part of two independent genes, or as a consequence of multiple allelism.

A detailed hematological delineation of this new entity is in progress. Physicochemical investigations to further characterize the new hemoglobin component are also in progress.

The assistance of the Hematology Clinic of the Children's Hospital of Detroit in many different aspects of these studies is gratefully acknowledged.

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† Assigned to the California Institute of Technology by the National Cancer Institute.
‡ Contribution No. 1457.

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BIOELECTRIC POTENTIALS AS A MEASURE OF RADIATION INJURY

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Communicated by Maurice Ewing, July 21, 1950

The criteria used to measure the biological effects of radiation by most workers as well as by the writer are in a sense purely biological in character, such as the size of the plant or embryo, the number of surviving colonies and the like. In 1942, Professor A. L. Romanoff of Cornell University worked with the writer on the effects of x-rays on the diameter of the developing blastoderm of the chick egg and also on the effects of x-rays on the bioelectric potential of the blastoderm.[1, 2] Dr. Romanoff and the writer were greatly impressed by the similarity of the curves representing the variation of the two parameters with dosage. The experiments seemed to indicate that the bioelectric potentials may be as reliable a measure of the effectiveness of radiation as the size of the embryo.

Because of the War, work was not resumed in this field until recently. In 1947 Jones[3] measured the variation of the bioelectric potential of seeds of corn and beans with x-ray dosage. The shape of the curves was exponential, typical of the variation of many relevant parameters with dosage. He also confirmed the results of Nelson and Burr[4] that the more vigorous strains of seeds have a higher potential. Since radiation affects the vigor of a plant, these results again indicated that potentials may be used as a measure of the effectiveness of radiation. Hunter[5] using wheat seedlings, which have easily measurable coleoptiles, found in some of his

measurements a similarity between the curves representing the variation of the length of coleoptile with x-ray dosage and the variation of the potential of the seed with the same dosage.

It seems worth while to examine carefully the relationship between the potential measurements and radiation injury, and the extent of validity of this relationship. Wheat seedlings were used in these experiments because of their conveniently measurable coleoptile lengths with which potential measurements may be compared.

The work of Carleson[4] and others established the fact that x-rays are most effective when the cells are in a certain stage of mitosis. It was of

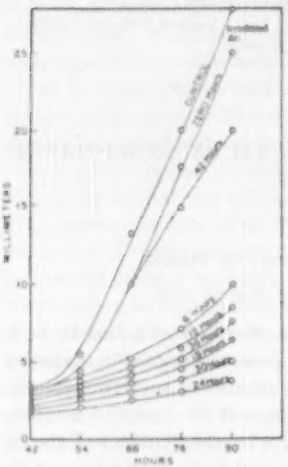

FIGURE 1

The length of coleoptiles as a function of time for seedlings given the same dose at different stages of growth.

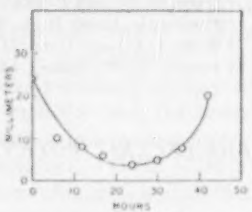

FIGURE 2

The length of coleoptiles at the same age as a function of time at which the radiation was applied.

interest in this work first to find the age of the seed at which most of the cells are in this stage, or the age most favorable for irradiation at room temperature. For this purpose groups of 30 seeds each were subjected to the same radiation dosage, 3000 r units, at 0, 6, 12, 18, 24, 30, 36, and 42 hrs. after immersion in nutrient solution. The lengths of coleoptiles were measured shortly after these appeared (about 48 hrs. after immersion in nutrient solution) and at 12-hr. intervals thereafter. Figure 1 shows the variation of the length of coleoptile as a function of time for each exposure. The effectiveness of radiation varies with the age at which the dose is administered, as was to be expected and is most easily shown by plotting the

length of coleoptile at a given stage of growth against the age at the time of exposure. Figure 2 shows such a curve for which the length measurements were made at 96 hrs. It is evident that radiation at 24 hrs. is most effective. Since the curves of figure 1 do not intersect, the same age for maximum effectiveness would have been obtained if the curve of figure 2 had been drawn for any other age after 72 hrs. However, the actual effectiveness (fractional length reduction) would have been different. It must be emphasized that this age for maximum effectiveness holds only for the room temperature of our laboratory (about 25°C.). The development of the seeds can be accelerated or retarded by a higher or lower temperature, and at a different temperature the radiation would be most effective at a different age.

Measurements of potential difference were made with an electrometer tube circuit. They were taken from the tip of the coleoptile to the seed

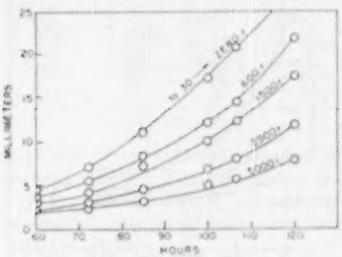

FIGURE 3

The length of coleoptiles of seeds subjected to various radiation doses as a function of time. Radiation administered at age of 24 hours.

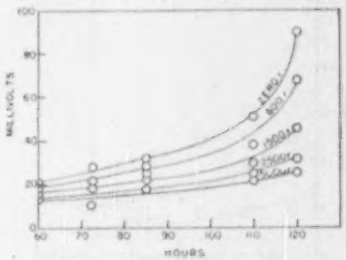

FIGURE 4

Potential of seeds subjected to various radiation doses as a function of time. Radiation administered at age of 24 hours.

coat. The experimental procedure is given elsewhere,[7] and will not be repeated here.

Figure 3 shows a series of curves of the length of coleoptile as a function of time of growth for several different radiation doses, all administered 24 hrs. after immersion in nutrient solution. Every point of the data presented here is the average of at least 30 seeds. For one reason or another, much of the data was retaken and many of the points on the curves shown represent an average of 60 seeds or more. Figure 4 gives the potential as a function of time for the same seeds. The most striking feature is the similarity of the two sets of curves, and both of them show clearly the diminution of the parameter with increasing dosage. The diminution of the two parameters is not in the same ratio. For example, at 72 hrs. the ratio of the length of coleoptile of control to that of highest exposure is 2 while at

120 it is 4. The ratio of potentials for the two periods is $1^1/_2$ and $3^1/_2$, respectively.

The effects of radiation on each of the two parameters is perhaps better shown in figures 5 and 6 where the length of coleoptile and the magnitude of the potential at a given stage of growth are plotted as a function of radiation.

There were a number of differences in the data on the measurement of the lengths of the coleoptiles and of the potentials which must be noted here. In the first place, some seeds showed a reversal of polarity, i.e., the coleoptile was positive with respect to the seed coat instead of being negative as in the great majority of the cases. The averages presented in figures 3–6 were obtained without regard to sign. The exclusion of the cases of opposite polarity would have affected the average of each group only

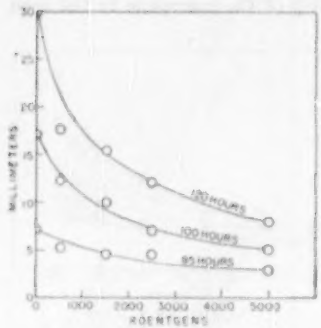

FIGURE 5

The length of coleoptiles at a given age as a function of dosage.

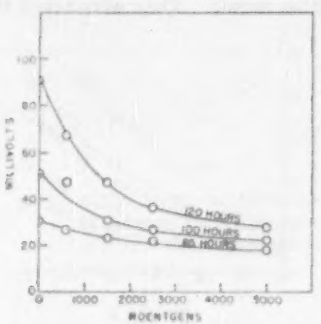

FIGURE 6

The potentials of seedlings at a given age as a function of dosage.

slightly and would have made little difference in the shape of the curve. It is interesting to note that the number of cases of opposite polarity decrease with increasing dose, being completely absent in groups subjected to heavy radiation doses.

Another extremely important difference is in the distribution of the magnitudes of each parameter within a given group. The lengths of coleoptiles of a given group are much more homogeneous than corresponding potential measurements, all length measurements being crowded close to the average. In each group there were a few length measurements which differed considerably from the average, but these few were clearly exceptions (giants or dwarfs; in the majority of cases dwarfs). If these are excluded, the range of variations within a given group is rarely greater than threefold.

This uniformity is not true at all for potentials. The individual values are much wider apart, a 20-fold variation within a given group not being uncommon, and all values seem to be represented. The exclusion of readings having a deviation four times the average deviation for a given group did not materially affect the average value of the potentials of the group. Figures 7 (*a*) and (*b*) show the distribution of coleoptile lengths for control and for a 3000 r dose, respectively, while (*c*) and (*d*) show the distribution of potentials for the same two groups. The variation in distribution of values of the two parameters is evident. It was noticed that the range of variation of values of coleoptiles or potentials were much smaller with increasing radiation dose. This is also demonstrated in figure 7.

The difference in distribution of the values of the lengths of coleoptiles

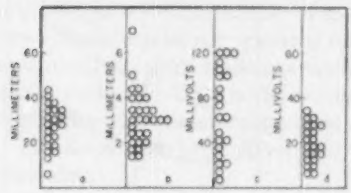

FIGURE 7

Distribution of (*a*) length of coleoptiles, control; (*b*) length of coleoptiles subjected to a dose of 3000 r; (*c*) potentials, control; (*d*) potentials of seeds subjected to a dose of 3000 r.

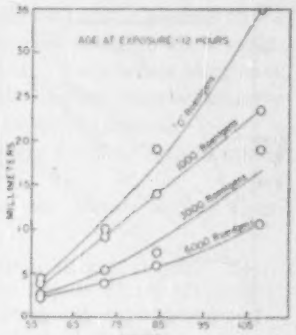

FIGURE 8

The length of coleoptiles of seeds subjected to various radiation doses as a function of time. Radiation administered at age of 12 hours.

and the magnitudes of potentials is to be expected. The length of the coleoptile is the sum total of cell activity over a considerable period of time. The larger the period the smaller is the range of variation of that parameter because individual differences seem to be ironed out. This is not true at all in the case of potentials. These measure the cell activity at a period immediately preceding the measurement or at the very time of measurement. Cell activity at a given time may vary widely, thus giving widely different values of the potential. A confirmation of this view was found in the fact that while with some seeds the galvanometer showed a steady deflection, with others the deflection did not remain steady at all, and fluctuated slightly. Another confirmation of this view was found in the fact that seeds with large coleoptiles did not necessarily give large values

of the potential. The cell activity even in a very vigorous seed may be small during a given time interval, thus giving small potential immediately after or during these intervals.

The large variation in the values of potentials may lead to erroneous conclusions if only a small number of observations are used. Figure 8 shows the variation of the coleoptile length of seeds subjected to different radiation doses 12 hrs. after immersion in nutrient solution, while figure 9 shows the values of the potentials of the same seeds. Figure 8 is very similar to figure 3, which shows the variations of coleoptile length with age at various exposures 24 hrs. after immersion in nutrient solution. On the other hand the first two curves of figure 9 are not at all similar to those of figure 4, being convex upward instead of concave. These curves would

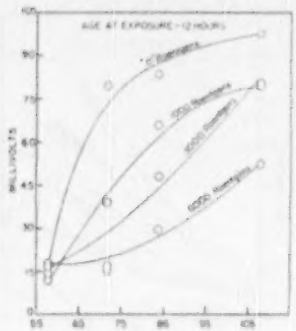

FIGURE 9

Potential of seeds subjected to various radiation doses as a function of time. Radiation administered at age of 12 hours.

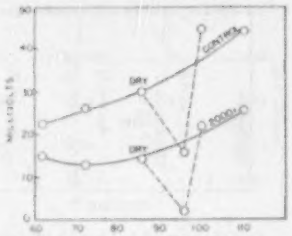

FIGURE 10

The potential of seeds subjected to dryness as a function of time.

give ratios of effectiveness of radiation much larger than those of coleoptile length and different from those for potentials mentioned earlier.

However, these two curves are truly exceptions, since out of some 40 or more sets of readings obtained in the course of the investigation these were the only ones which were so much out of line with the rest. On the basis of probability, such curves are to be expected if the number of specimens used is small, illustrating the danger of making conclusions from potential measurements of a few specimens. This naturally limits the usefulness of potential measurements for measuring radiation injury. On the other hand bioelectric potentials are very sensitive to temporary changes in the condition or environment of the organism. A lucky mishap brings this out very clearly. Because of neglect a tray containing groups

of seeds subjected to various radiation doses was not watered at a certain period of growth. Readings of the lengths of coleoptiles and of the values of the potentials were continued after the accident was noted, and after the tray was watered. The lengths of coleoptiles measured at the end of the "dry" period showed no appreciable departure from the curve. However, the potential showed a violent departure. Figure 10 shows the potential for only two of the groups of seeds, control and 2500 r exposure, as a function of time in the nutrient solution, the curves for three other exposures following exactly the same pattern. It is evident that the potentials suffered a very large drop.

It is reasonably safe to say that the average value of the potential of specimens is as good as measure of radiation injury as any other parameter, providing an adequate number of specimens are used. It is possible that a series of readings on one specimen may give as good an indication of radiation injury as a single reading of many specimens. Work is now being done on this problem. Work is also in progress on the length of time between the application of the radiation stimulus and the bioelectric response.

Thanks are due to B. Kivel and A. Rosenberg for their help in taking these data.

This work was done under the auspices of the Atomic Energy Commission.

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THE LOCI OF ACTION OF ULTRA-VIOLET AND X-RADIATION, AND OF PHOTORECOVERY, IN THE EGG AND SPERM OF THE SEA URCHIN

BY HAROLD F. BLUM,* J. COURTLAND ROBINSON AND GORDON M. LOOS

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Communicated by E. N. Harvey, September 29, 1950

Given in moderate doses, both ultra-violet radiation and x-ray delay cell division (cleavage) of the eggs of the sea urchin *Arbacia punctulata*. The present communication summarizes experiments which indicate that the locus of this action is, in both cases, associated with cell nuclei, as contrasted to cell cytoplasm. There is recovery from the effect of the radia-

tion, as indicated by return toward the normal rate of cleavage. Following treatment with ultra-violet radiation, recovery is accelerated by illumination with "visible" radiation. Our experiments indicate that this photorecovery has its locus in the cytoplasm of the egg. In the case of x-ray, visible radiation appears to be without effect on the recovery of cleavage rate.

We are concerned here with the conclusions that may be reached by summarizing the results of a variety of experiments. Some of these have already been discussed in other papers, which include descriptions of the general method used.[1] Those results which have been newly added will be described in greater detail elsewhere.

Locus of the Delay of Cleavage by Ultra-Violet Radiation.—Advantage has been taken of the fact that the eggs of *Arbacia* can be separated by centrifugation into nucleate and enucleate halves, adding to the number of experimental combinations with which one may work. The whole eggs may be exposed to ultra-violet radiation,[2] either before or after fertilization with normal sperm. The same procedure may be followed with the

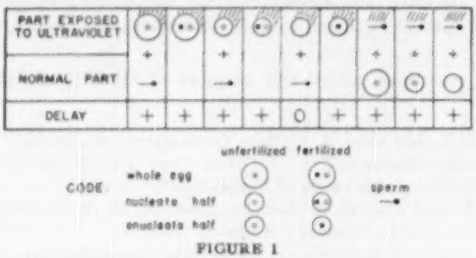

FIGURE 1

nucleate or enucleate halves. Or, the sperm may be exposed to the ultra-violet radiation before it is used to fertilize the eggs or halves. Various combinations are indicated in figure 1, which also summarizes the results with regard to the delay of cleavage. Delay is observed in all cases except when the enucleate half is exposed to ultra-violet radiation before fertilization with normal sperm. This is also the only case in which the part that receives the radiation contains no nucleus. If the sperm nucleus is introduced into the enucleate half by fertilization before the exposure, or if the sperm itself is exposed to the ultra-violet radiation, delay of cleavage results. The conclusion seems obvious that the locus of action of the radiation is the nucleus or something closely associated with it. Reasons will be discussed shortly for thinking that nucleoprotein is the substance absorbing the ultra-violet radiation in the primary photochemical act leading to delay of cleavage.

Locus of Photorecovery.—After the initial delay by ultra-violet radiation, there is a gradual return toward the normal cleavage rate. This recovery

process is greatly accelerated by illumination with "visible" radiation.[3] To determine the locus of the photorecovery process, experimental combinations similar to those already described were employed. The results are summarized in figure 2. In the case of the enucleate half exposed to ultra-violet before fertilization, the presence or absence of photorecovery cannot be demonstrated because there is no delay of cleavage. Photorecovery was observed in all other cases except that of the sperm before introduction into the egg. In such experiments, a sample of sperm was exposed to ultra-violet radiation, and then divided into two portions, one of

FIGURE 2

which was illuminated with visible radiation, while the other was placed in the dark. One hour later, samples of normal eggs were fertilized, one with the illuminated sperm, the other with the sperm that had been kept in the dark. The two samples of eggs underwent cleavage at the same time. On the other hand, when sperm which had been exposed to ultra-violet radiation were used to fertilize normal eggs, photorecovery was observed. That is, those fertilized eggs subsequently illuminated with visible radiation recovered more rapidly than those kept in darkness. Nucleate or enucleate halves may take the place of the whole eggs in this experiment. Thus, it

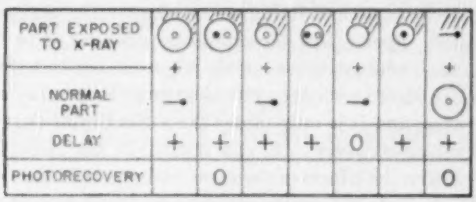

FIGURE 3

appears that egg cytoplasm is essential for the photorecovery process.

The sperm lacks altogether the power to recover, whether in light or darkness. If two samples of the same preparation of sperm are exposed to doses of ultra-violet radiation identical in amount, but applied one hour later in the one case than in the other, there is no difference in the resultant amount of cleavage delay when normal eggs are fertilized with these samples of sperm. Thus, the ability to recover, whether in light or dark, is associated only with egg cytoplasm.

Locus of Delay of Cleavage by X-Ray.—Experiments with x-ray are summarized in figure 3. The results parallel those with ultra-violet radiation

in showing that the effect is associated with the nucleus. In contrast, however, visible radiation was not found to accelerate recovery from x-ray, indicating that this agent acts in a fundamentally different way from ultra-violet radiation. Figure 3 has been amplified by including, together with our own results, some of those reported by Henshaw. Henshaw's extensive experiments on unfertilized eggs[4] we have not repeated.[5] He found, as did we, that when exposed to x-ray before fertilization, nucleate halves showed delay of cleavage, but enucleate halves did not.[6] He mentions none of the other experimental combinations with nucleate and enucleate halves, which we report. Henshaw found, parallel to our experiments with ultra-violet, that sperm exposed to x-ray showed no power of recovery.[6]

Discussion.—The photorecovery after ultra-violet radiation, manifested by the *Arbacia* egg, seems in all ways parallel to the "photoreactivation" in fungi and bacteria, which has been the subject of a number of papers since it was reported by Kelner[7] a year ago. The phenomenon has been demonstrated in widely separated animal and plant forms.[8] The experiments described herein seem to parallel closely those of Dulbecco[9] with bacteriophage and *Escherichia coli*, so special reference will be made to that work. The wave-lengths effective in photorecovery and photoreactivation are the same. In both cases, the visible light has no demonstrable effect unless some part of the system has been previously exposed to ultra-violet radiation.[1, 9] Indeed, these aspects are characteristic of the phenomenon wherever it has been studied. If the sperm is taken to be comparable to bacteriophage, and the egg to be comparable to *E. coli*, the parallelism is revealed. Sperm, like bacteriophage, does not show recovery from the effect of ultra-violet radiation. Only when the irradiated sperm or bacteriophage is associated with egg cytoplasm or *E. coli*, respectively, does recovery take place, and it is only under these conditions that light plays a role in accelerating recovery. *E. coli* itself, like the *Arbacia* egg, shows photorecovery from the effects of the ultra-violet radiation.

Since the bacteriophage is virtually pure nucleoprotein, it seems almost certain that this substance is the absorber of the ultra-violet radiation. It is also reasonable to think that nucleoprotein of the nucleus is again the absorber of ultra-violet radiation in the primary photochemical act initiating cleavage delay. It is important to point out that our experiments do not preclude action of ultra-violet radiation on parts of the cell other than the nucleus, but only indicate that such action does not affect the rate of cleavage of the egg.

Neither sperm nor bacteriophage can "repair" the "damage" done by ultra-violet radiation, this being a function of egg cytoplasm or *E. coli*, respectively. Moreover, neither the sperm nor bacteriophage is strictly a self-reproducing system. The bacteriophage multiplies only in association

with the host cell, which is presumably essential for synthesis of nucleoprotein. Similarly, the sperm nucleus is associated with cell division and the synthesis of nucleoprotein only after it is brought into the presence of egg cytoplasm. In attempting to explain this parallelism, let us postulate that ultra-violet radiation alters the nucleoprotein, bringing about some minor change in configuration that can be reversed by the synthetic processes carried out by systems generally present in the cytoplasm,[10] but absent in the case of the sperm. It is tempting to associate the repair after exposure to ultra-violet radiation with the synthesis of nucleoprotein; and since the latter no doubt involves endergonic processes, to regard the repair, too, as endergonic. It is tempting, further, to think that visible radiation accelerates the repair by contributing energy to this endergonic process. But this may be carrying speculation beyond the point of profit, unless further evidence can be supplied.

Acknowledgments.—It is a pleasure to acknowledge the numerous helpful suggestions of Dr. E. B. Harvey during the course of this investigation.

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[2] The effective wave-lengths used in our experiments were those of the mercury lines from 0.27 μ to 0.313 μ.

[3] The wave-lengths effective in photorecovery range from the near ultra-violet into the blue; the long wave-length limit being approximately 0.5 μ.

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THE IMMEDIATE DEPENDENCE OF THE ACTION OF A SPECIFIC GENE IN DROSOPHILA MELANOGASTER UPON FERTILIZATION

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In *Drosophila melanogaster*, a particular gene complex composed of a semidominant mutant, erupt (*er*), together with a semidominant suppressor of erupt (*Su-er*), was discovered by the senior author[1] and found to be widely distributed in laboratory stocks and wild populations of this

species.[3] (The erupt phenotype consists of a large eruption of non-faceted material usually through the center of one or both eyes. Sometimes this structure bears bristles. In weaker manifestations of erupt the facets in the central region of the eye are merely disarranged. There may be a small extra bristle on the anterior margin of the eye, dorsal to the antenna.) The two loci are in separate linkage groups and segregate in characteristic Mendelian fashion. The loci are demonstrably not strictly duplicate loci, for the two reciprocal genotypes *Su-er*$^+$ *Su-er*$^+$; *er*$^+$ *er*$^+$ and *Su-er Su-er;*

TABLE 1

THE EFFECTS OF X-RAYS APPLIED AT VARIOUS AGES IN BLOCKING THE ACTION OF THE SUPPRESSOR-ERUPT GENE

AGE AT RADIATION (HRS. POST-LAYING)	TOTAL EGGS	TOTAL ADULTS ENCLOSED AND CLASSIFIED	EYE PHENOTYPE: NORMAL	WEAK ERUPT	EXTREME ERUPT	ERUPT, %: TOTAL	ERUPT, %: EXTREME	DIFFERENCE AND S. E. DIFF.
1 ± 0.25	1973	63	2	7	54	96.8	85.7	0.94 ± 0.05
Control	491	424	413	10	1	2.6	0.24	
3 ± 0.5 (*a*)	1066	106	40	38	28	62.3	26.4	0.40 ± 0.06
Control (*a*)	165	158	123	34	1	22.2	0.6	
3 ± 0.5 (*b*)	2083	41	4	7	30	90.2	73.2	0.87 ± 0.06
Control (*b*)	260	241	233	8	0	3.3	0.0	
5 ± 1	1063	34	0	9	25	100.0	73.5	0.99 ± 0.06
Control	323	276	274	2	0	0.73	0.0	
10 ± 1	310	47	2	14	31	95.7	65.9	0.95 ± 0.07
Control	150+	150	149	1	0	0.7	0.0	
15 ± 1	529	36	0	5	31	100.0	86.1	0.78 ± 0.09
Control same as for 3 ± 0.5 (*a*)								
18 ± 1	1488	225	27	50	148	88.0	65.8	0.84 ± 0.05
Control	255	232	223	9	0	3.9	0.0	
24 ± 1 (Larvae)	342	147	2	13	132	98.6	89.8	0.99 ± 0.05
Control	260	246	246	0	0	0.0	0.0	
LARVAE (HRS. POST-HATCHING)								
55	292	217	38	28	151	82.5	69.6	0.82 ± 0.04
Control	390	359	355	4	0	1.1	0.0	
75	175	160	115?	7?	38	28.1	23.8	0.25 ± 0.04
Control	235	209	202	7	0	3.4	0.0	0.025 ± 0.02
100	140	120	113?	7?	0	5.8	0.0	

er er, which would be identical if the two loci were duplicates (*Su-er* being equivalent to *er*$^+$ and *Su-er*$^+$ to *er*), do not respond alike to a certain specific environmental agent. If embryos at an age of 10 hours are given an x-ray dose of 1000 r units, the eyes of the enclosing adult flies of the first genotype given above are quite normal, whereas those of the second genotype express a strong manifestation of the erupt character in from 90 to 100% of the treated individuals. The effect of x-rays on the particular genotype *Su-er Su-er; er er* was attributed to a specific blocking of some phase of the action of the suppressor gene.

In order to determine the critical period during which an x-ray treatment will inactivate the effect of this suppressor-erupt gene, doses of 1000 r units were applied to embryos aged 1 hour ± 1/4 hour, 3 hours ± 1/2 hour, 5 hours ± 1 hour, 10 hours ± 1 hour, 15 hours ± 1 hour, 18 hours ± 1 hour, and 24 hours ± 1 hour. These treatments covered the entire embryonic period from 1 hour after the egg is laid to a time when the eggs have hatched into first instar larvae. All cultures were kept at 25 ± 1°C. As table 1 and figure 1 show, the inactivation of the suppressor-erupt effect was indistinguishable at all of these ages from that exerted upon embryos 10 hours old and was virtually complete.

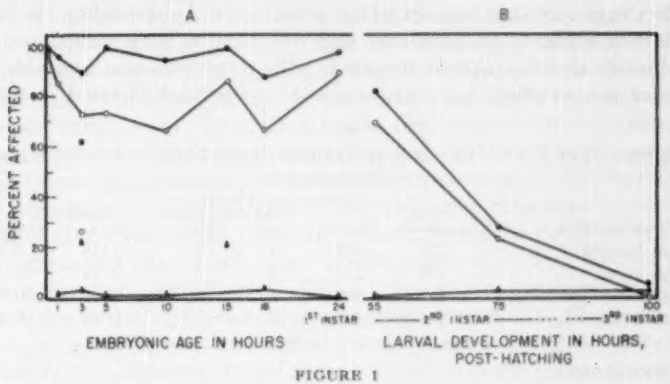

FIGURE 1

Percentages of individuals manifesting erupt eyes when treated with 1000 r units of x-rays at various ages. ●, per cent manifesting erupt to any degree, x-rayed series; ○, per cent manifesting extreme erupt, x-rayed series; ▲, per cent manifesting erupt to any degree, untreated control series.

Controls in each case are from eggs laid by the same parents who laid the eggs treated with x-rays. All control series are homogeneous except for series (a) at 3 ± .5 hours. In this series, the percentage of weak erupt was very high, and simultaneously the inactivation of the suppressor in the treated individuals was far less complete than usual, although the control and treated series were still significantly different. The test at this age was therefore repeated [series (b)] with results quite in line with those obtained in all the other series. In figure 1, the values obtained in series (a) at 3 hours are plotted, but have not been used in drawing the curves.

Extension of the tests into the larval period showed that the inactivation of the suppressor-erupt effect is still nearly maximal 55 hours after the larvae have hatched from the egg, at a time well into the second larval instar. At 75 hours of larval development, in the third instar, the effectiveness of the x-ray dose has diminished very considerably, and by 100 hours of larval age it has almost completely disappeared.

Disarrangement of the facets is so general in individuals treated with x-rays at 75 or 100 hours of larval life that classification of weak erupt could only be based on the presence of the extra bristle on the anterior dorsal margin of the eye. The (?) signs in table 1 in these two series indicate this inability to classify erupt on the same basis as in the other series.

The effectiveness of the x-ray treatment so very early in the embryonic period led us to make a further effort to obtain and treat eggs as young as they could possibly be collected in sufficient numbers and also to see whether the treatment of the spermatozoa and oöcytes prior to fertilization might be effective. It proved possible to collect and treat eggs at an average age of 8 minutes ± 8 minutes after they were laid. Care was taken to collect eggs only from females laying actively and, by discarding the first collection made, to eliminate any eggs which might have been stored in the female after having been fertilized. The x-ray treatment (see table 2) proved just as effective in eliminating the suppression of erupt in these

TABLE 2

THE EFFECTS OF X-RAYS ON THE SUPPRESSOR OF ERUPT PRIOR TO AND SUBSEQUENT TO FERTILIZATION

		PHENOTYPE OF EYES			ERUPT, %	
A. X-RAYED 1000 R	TOTAL CLASSIFIED	NORMAL	WEAK ERUPT	EXTREME ERUPT	TOTAL	EXTREME
Adult females	339	335	4	0	1.18	0.0
Adult males	279	277	2	0	0.72	0.0
Adult males and females	406	402	4	0	0.99	0.0
Control (not x-rayed)	440	436	4	0	0.91	0.0
[$\chi^2 = 0.364$ $n' = 3$ $P = 0.95 - 0.90$]						
B. X-RAYED 1000 R						
Embryos: 8 ± 8 minutes	49	0	1	48	100.0	98.0
Control (not x-rayed)	227	226	1	0	0.44	0.0

newly laid eggs as it had in older embryos, 98% of the flies hatching from them manifesting strongly erupt eyes. On the contrary, neither radiation of spermatozoa nor of oöcytes separately, nor of both together, produced any inhibition of the suppressor effect. This was true even when the dosage of x-rays was increased to 2500 r units. In these first treatments of the germ cells before fertilization, the treatments were given separately to mature males and females, which were thereafter mated. In order to shorten the time between treatment and fertilization, we next treated with x-rays females which had already been inseminated. Such females would carry, at the time of treatment, one or two already fertilized eggs in utero, but most of the eggs laid would come from oöcytes and spermatozoa treated prior to fertilization. As expected, a very few erupt flies were produced, and in cultures from single females these were distributed as follows: 16 with no erupt offspring; 6 with 1 erupt; 2 with 2 erupt; 1 with 3 erupt. The overwhelming majority of offspring showed complete suppression of erupt.

The difference between the two series, the one treated prior to fertilization and the other as soon as possible subsequent to it, is very striking and statistically significant far below the 0.001 level ($\chi^2 = 980.7$; $n' = 2$). Since, according to the studies of Huettner,[3] meiosis of the egg is arrested at metaphase of the first meiotic division until fertilization occurs, the completion of meiosis and the formation of the polar bodies must occur within the 8 minutes before the fertilized eggs were treated. According to Rabinowitz,[4] the fertilized egg of Drosophila takes 23 ± 1.72 minutes at 24°C. to reach the first division of the cleavage nuclei, and at 15 ± 1.21 minutes it is either in telophase of the second maturation division or at the conjugation of the pronuclei. It therefore appears that the 8-minute-old embryos treated in this experiment could hardly have reached the first cleavage division, on the average. This appears to signify that as the entry of the sperm activates the egg to form its polar bodies, so too it activates it to produce at least one specific gene-initiated substance or morphogenetic system, namely, that one which is due to the presence of the suppressor-erupt gene and which is inactivated by x-rays. Still, this specific reaction that follows immediately upon activation of an insect egg by fertilization is far from being obviously related to such activation phenomena as an increased rate of respiration, production of acid, superficial cytolysis, release of calcium ion, etc., which occur in echinoderm eggs upon fertilization (see reviews by Lindahl[5] and Tyler[6]). One may suppose, however, that the action of the suppressor-erupt gene may, according to the theory of Runnström,[7] involve a more intimate contact between catalysts and substrate or the liberation of a mediator. Isolation of the chemical system which is inactivated by x-rays would clarify this problem. Meanwhile it remains uncertain whether the postulated inactivated substance is to be regarded as enzyme or substrate.

To our knowledge, this is the most precise determination yet made of the time at which the action of a specific gene is determined. The reasoning upon which this is based is as follows: (1) Production of the complete blocking effect by exposure to x-rays at any age up to 24 hours, by which time there are great numbers of nuclei, and furthermore the failure of the blocking effect to be transmitted through the germ line to the next generation, alike indicate that the effect of the x-rays is not at all to cause the suppressor gene itself to mutate, but is rather to inactivate some product of this gene. The action of the x-rays is direct and cannot in some way be stored so as to produce a later effect on a substance or morphogenetic system that was not present at the time of irradiation. This follows from the fact that treatment of mature oöcytes and spermatozoa is completely ineffective, whereas the treatment of just fertilized eggs is completely effective. There is absolutely no carry-over. Moreover, the target altered in nature by the irradiation is probably to be identified as a product of the

suppressor gene, for it is not present in the absence of the suppressor gene, i.e., in the genotype *Su-er*$^+$ *Su-er*$^+$; *er*$^+$ *er*$^+$. It could however be an essential substrate for the suppressor gene. (2) The complete blocking of the suppressor action at any age from 8 minutes up to 24 hours shows that the x-rays destroy some substance or morphogenetic system which cannot be replenished or repaired. The simplest explanation is that a specific gene product or substrate is formed in full amount prior to the earliest time at which complete inactivation can be obtained and that it persists unmodified in amount for so long a time as it is still possible to obtain complete inactivation by means of the x-rays. In other words, the gene product or substrate here involved is present in full amount within 8 minutes after fertilization and persists unchanged in amount until some time after the larva has hatched from the egg. (3) During the second and third larval instars the inhibitory effect of x-rays on the action of the suppressor of erupt diminishes progressively. This indicates either that the primary gene product or substrate is gradually being used up or that the morphogenetic system in which the product (or substrate) participates has advanced beyond the stage at which the product or substrate can modify it.

It might be supposed that the suppressor gene exerts its primary effect before the egg is fertilized but that its product is not susceptible to x-rays until after it has been in some way changed through the activation of the egg by fertilization. To clear up this question, females carrying the mutant *er* but not its suppressor (*Su-er*$^+$/*Su-er*$^+$; *er*/*er*) were x-rayed with the same dose used previously and then crossed with males of the genotype *Su-er*/*Su-er*; *er*/*er*. The flies obtained (n = 839) were not significantly different from the controls in the percentage of erupt. This proves that the suppressor gene acts subsequent to its introduction by fertilization.

It might also be questioned whether the suppressor gene acts as promptly after fertilization when it is introduced through the spermatozoon as when it is already present in the oöcyte. Experiments to determine this point are now under way. Preliminary data show that it has already produced its primary x-ray-sensitive effect by the time the embryo is 5 hours ± 1 hour old.

The fact that a specific reaction system which affects the conformation of the eye can be detected a few minutes after fertilization, although completely lacking prior to that event, makes it plausible to describe this determination of the initiation of the action of a specific gene as an instance of a *primary* gene action. It belongs in a different category than those determinations of the sensitive periods during which temperature or other modifying agents affect certain gene-controlled processes or produce phenocopies. As Goldschmidt[5] has emphasized, such sensitive periods characteristically occur during the middle to late larval and pupal periods,

at a time when the affected structures are differentiating from their anlagen (pp. 3–51). Steinberg,[9] Vogt,[10–13] Begg and Sang,[14] and others have shown, moreover, that such a sensitive or effective period by no means necessarily corresponds to the time of action of the gene. Different effects of the same gene may even have different temperature-sensitive periods. The specific gene action demonstrated here, however, not only occurs long before even the imaginal disks which give rise to the eyes are laid down but even before cleavage has progressed beyond a single division.

Indications of the precise time of action of certain genes have generally been seen in cases of genetic mosaicism, both in plants and in animals. Such situations differ in an essential respect, however, from that analyzed in the present case. The production in an organism of adjacent, sharply bounded areas contrasting in phenotype is dependent upon the fact that the individual was originally heterozygous (or mosaic) as a whole, or has become heterozygous through mutation in some part of its body. What is actually observed in the mosaic is the product of an event giving rise to a segregation of alleles and consequently to a difference of genotype in particular cell lineages. It is this event which is precisely determinable in ontogeny, and not necessarily the normal time of action of a specific gene. In a case of somatic segregation, for example, at whatever stage in ontogeny the segregation occurs, the genes exhibiting mosaicism might act at any time subsequent to the segregation. Only for cases of somatic segregation late in ontogeny is the time of action of the said genes therefore rather precisely determinable. Even so, there is an obstacle to a rigorous conclusion that the time of gene action is here precisely determinable. The recessive allele may in fact have been acting long before the segregation which permits it to escape from its dominant partner and so to manifest itself. Even the dominant allele may have been acting prior to the segregation, if its primary product (after the fashion of kappa in *Paramecium aurelia;* cf. Sonneborn[15]) is unable to persist in the cell in the absence of the gene itself, so that the primary gene product might disappear before the arrival of that time at which it normally would enter the specific morphogenetic system it modifies. The same reasoning holds, *pari passu*, in cases of mosaicism produced by somatic mutation. On the other hand, all such difficulties of interpretation are obviated in the analysis of the action of the suppressor-erupt gene, because at all stages the same genotype is present. It is particularly to be emphasized that the unreduced and metaphase I oöcytes which were irradiated with x-rays were identical in genotype with the fertilized eggs which were treated. The spermatozoa likewise possessed the same genotype, but of course were haploid.

A consideration of this case makes one wonder how many other genes actually initiate action just after fertilization, and to what extent different genes really come into action at different stages throughout the course of development.

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GENE INDUCED MUTATION OF A HERITABLE CYTOPLASMIC FACTOR PRODUCING MALE STERILITY IN MAIZE

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Earlier studies[1-3] have shown that the recessive gene iojap (*ij*) in maize induces irreversible mutation of plastid primordia. Further work indicates that this gene not only affects plastids but also produces mutation of a second cytoplasmic factor which is concerned with development of the male gametophyte (pollen). When a homozygous iojap plant is used as the egg parent in a cross with a normal green individual the F_1 progeny often consists of white and green-white striped plants in addition to varying proportions of the expected wholly green class. The reciprocal cross invariably yields only normal green offspring. These white and striped plants arise from plastid mutation induced by the iojap gene. The white seedlings come from those egg cells with only mutated plastids, the striped plants from eggs with both normal and mutated plastids, and the green individuals from a cytoplasm with unaffected plastids.

The evidence for the mutation-inducing effect of iojap on a second cytoplasmic factor is as follows. Varying degrees of pollen abortion are found in homozygous iojap plants. If iojap plants are used as the pollen parent in crosses with normal, male-fertile individuals, the F_1 progenies contain only green, wholly male-fertile plants. However, in the reciprocal cross plants which are either completely or partially male-sterile may occur in addition to normal male-fertile individuals. When these male-sterile F_1

plants were pollinated by unrelated male-fertile individuals, the ensuing backcross populations have consisted solely of male-sterile offspring. The failure of the pollen parent to affect the heredity of the male-sterile character indicates that its transmission is cytoplasmic. More meaningful is the fact that the male-sterile phenotype owes its inception to the action of the iojap gene, as is also true for mutation of plastid primordia.

Since the mutation-inducing effect of iojap on plastids resembles that on the cytoplasmic particle concerned with pollen development the question arises whether or not both of these phenotypic characters may be due to the same cytoplasmic factor. It can be stated with some assurance that this is not true. Mutation of the plastid primordia is independent, or largely so, of that of the cytoplasmic factor determining male sterility, for if it were not then only the white plants would, if viable, be male-sterile, the tassel branches on white sectors of striped plants would have aborted pollen while those from green sectors should have normal pollen, and the green siblings should be male-fertile. Such is not the case. Evidence of the particulate nature of this cytoplasmic factor is the occurrence of partially male-sterile plants with diverse percentages of pollen abortion. It is believed that these partially sterile plants arise from egg cells with a cytoplasm containing both normal and mutated particles. Completely male-sterile plants presumably possess only mutated particles while male-fertile individuals have non-mutated particles.

If, as we believe, the cytoplasmic particles determining male sterility are mitochondria, these observations are of some interest in connection with the problem of the differentiation of cytoplasmic particles. Plastids have a genetic continuity; such a fundamental property has been conjectured but never demonstrated for mitochondria. Further, in meristematic cells of maize it is difficult to distinguish between mitochondria and plastid primordia. The statement[4] that plastids arise from mitochondria suggests there is no basic difference between them. It should be emphasized that the postulated role of mutated mitochondria in producing pollen abortion is hardly a demonstrated fact and some other cytoplasmic component may well be involved. However, if our surmise is correct, these investigations provide the first evidence of not only the genetic continuity of mitochondria but also of a fundamental difference between them and plastid primordia. In any event the iojap gene has been shown to induce irreversible mutation of some cytoplasmic constituent other than plastid primordia, and this is in itself of more than passing interest. Needless to say this problem will receive our further attention.

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DIFFERENTIAL REJOINING AS A FACTOR IN APPARENT SENSITIVITY OF CHROMOSOMES TO X-RAY BREAKAGE*

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It has previously been shown[1, 2] that the sensitivity of Trillium chromosomes to x-ray fragmentation varies greatly with the stage of meiosis irradiated. Irradiation of certain stages yields up to fifty times more breakage than that obtained with the same dosage at other stages.[2, 3] The reason for such changes in radiosensitivity of the chromosomes is not known, but it seemed plausible that a comparison of the relative number of certain types of aberrations produced at a highly sensitive stage with those produced at a stage of low sensitivity might yield pertinent information. More specifically, it was decided that aberrations following irradiation at first meiotic metaphase and early post-meiotic interphase would be studied. These are stages of high and low sensitivity, respectively, the former being approximately fifty times as sensitive to breakage as the latter.[2, 3]

Since the work involved in a complete analysis of all aberration types in many hundreds of cells would be prohibitive, scoring was done for only three types; acentric fragments, dicentrics and rings. They were scored at microspore anaphase. The number of fragments served as a rough index of sensitivity and the number of dicentrics and rings as an index of rejoining of broken ends. It was thus possible to compare the amount of rejoining at different stages relative to the sensitivity of that stage. Such a comparison has given information which indicates that rejoining of broken ends probably can and does affect apparent radiosensitivity of chromosomes to x-ray breakage.

Materials and Methods.—Rhizomes of *Trillium erectum* L. obtained from commercial sources were handled as previously described.[4] Test slides were made from one or more anthers just before x-raying to determine the stage. Immediately after treatment the plants were returned to the cold room and left there until they reached the appropriate stage of microspore development. This varied from about five to ten weeks, depending upon the stage and dosage. Permanent propionic or acetocarmine smears mounted in euparal or diaphane were used exclusively. The details of the method used have been published elsewhere.[4]

It should be noted that in *Trillium erectum* L. there is no regular interphase following first division. The first anaphase chromosomes rearrange themselves, and second division follows directly. Post-meiotic interphase is therefore the first interphase to occur after irradiation at first

metaphase. It should also be remembered that in Trillium (and other material) irradiation of metaphase chromosomes does not produce immediate breaks but only potential breaks.[1] True breaks are generally not visible until after the chromosomes have passed through an interphase stage and are approaching metaphase of the next division.

Irradiation was from a Coolidge type tube with Tungsten target operated at a peak of 160 kv. and 10 ma. The half value layer was approximately 0.3 mm. of Cu and its effective wave length was 0.23 Å. Distance from the target was constant in all experiments and time was varied to control dosage. Measurements of dosage were made on an integrating

TABLE 1

SUMMARY OF DATA FROM MATERIAL IRRADIATED AT FIRST MEIOTIC METAPHASE AND SCORED AT MICROSPORE ANAPHASE (ARRANGED IN ORDER OF FRAGMENTS PER 100 CELLS[a])

PLANT AND SLIDE[b]	DOSAGE IN ROENTGENS	NO. OF ANAPHASES ANALYZED	NO. OF ANAPHASE FRAGMENTS		DICENTRICS[c] OBSERVED	DICENTRICS[c] AND RINGS	
			OBSERVED	PER 100[a] CELLS		OBSERVED	PER 100 CELLS[a]
367PO	50	50	53	106.0	1	2	4.0
166FH	25	217	290	133.6	9	14	6.5
166BEDC	25	211	284	134.6	5	10	4.7
144D	50	100	149	149.0	13	15	15.0
143PQ	50	120	179	149.2	20	21	17.5
144N	50	60	96	160.0	9	10	16.7
144E	50	140	227	162.0	29	31	22.1
2375G	100	25	79	316.0	17	21	84.0
917C	200	78	274	351.3	49	76	97.4
2375F	100	25	108	432.0	22	28	112.0
153D	50	32	157	490.6	31	37	115.6
2375P	100	25	143	572.0	42	48	192.0
2150POQ	100	38	246	647.4	43	53	139.5
117E	100	40	263	657.5	33	50	125.0
Total		1161	2548	...	323	416	...

[a] Cells here refer to microspores.

[b] Each number represents a plant, each letter a slide.

[c] Dicentric rings were scored as rings. Tricentrics were scored as 2 dicentrics, etc.

type of dosimeter calibrated against a Victoreen r-meter of known accuracy.

Results.—Data were collected from buds irradiated with 25, 50, 100 and 200 r at meiotic first metaphase (table 1) and with 50, 100, 200, 400, 800 and 1000 r at early post-meiotic interphase (table 2). This range of dosages, plus a certain amount of inherent variability in susceptibility of plants to x-rays, has given a wide range of induced fragment frequencies. The data within each table are arranged in order of ascending fragment frequencies.

Ring-shaped aberrations were scored as rings only when large enough to

TABLE 2

SUMMARY OF DATA FROM MATERIAL IRRADIATED AT EARLY POST-MEIOTIC INTERPHASE AND SCORED AT MICROSPORE ANAPHASE (ARRANGED IN ORDER OF FRAGMENTS PER 100 CELLS)

PLANT AND SLIDE[a]	DOSAGE IN ROENTGENS	NO. OF ANAPHASES ANALYZED	NO. OF ANAPHASE FRAGMENTS		DICENTRICS[c] OBSERVED	DICENTRICS[c] AND RINGS	
			OBSERVED	PER 100 CELLS[b]		OBSERVED	PER 100 CELLS[b]
889E	50	100	9	9	2	2	2
197E	50	100	11	11	2	2	2
513N	50	100	12	12	3	3	3
182FD	50	100	13	13	3	4	4
182NO	50	100	13	13	2	2	2
929O	50	50	7	14	1	1	2
185D	100	223	37	16.6	3	8	3.6
688O	100	111	19	17	2	2	1.8
759R	100	100	18	18	1	2	2
919E	50	100	20	20	1	1	1
467FG	50	100	20	20	4	4	4
513C	50	50	5	10	0	0	0
179C	100	153	31	20.3	3	7	4.6
715CB	100	100	21	21	0	0	0
197G	50	100	21	21	0	0	0
579-O	50	100	24	24	2	2	2
688R	100	80	23	25.8	0	3	3.4
119D	100	82	25	30.5	7	11	13.4
661DEF	100	100	32	32	5	5	5
649-O	100	100	34	34	5	5	5
715N	100	100	35	35	4	4	4
179B	100	42	15	35.7	2	2	4.8
68F	200	100	127	127	31	39	39
378BCDE	400	100	130	130	32	44	44
832CZD	200	100	131	131	9	18	18
378BE2	400	100	141	141	31	37	37
$88B_1B_2$	200	100	145	145	23	26	26
211C	200	200	324	162.0	42	53	26.5
329C-351CD	200	50	137	274.0	37	58	116.0
375D	800	80	246	307.5	72	74	92.5
480C-E	400	58	186	320.7	34	55	94.8
75BCD	200	62	217	350.0	24	36	58.1
371BCFG	1000	90	318	353.3	41	58	64.4
444EFG	200	64	232	362.5	78	110	171.9
377BCD	800	101	412	407.9	96	156	154.5
377M	800	50	220	440.0	50	64	128.0
Total		3455	3411	...	652	898	...

[a] Each number represents a plant, each letter a slide.

[b] Cells here refer to microspores.

[c] Dicentric rings were scored as rings. Tricentrics were scored as 2 dicentrics, etc.

have an unmistakable hole in the center. Questionable cases were not scored. Dicentric rings were counted as rings only and not included

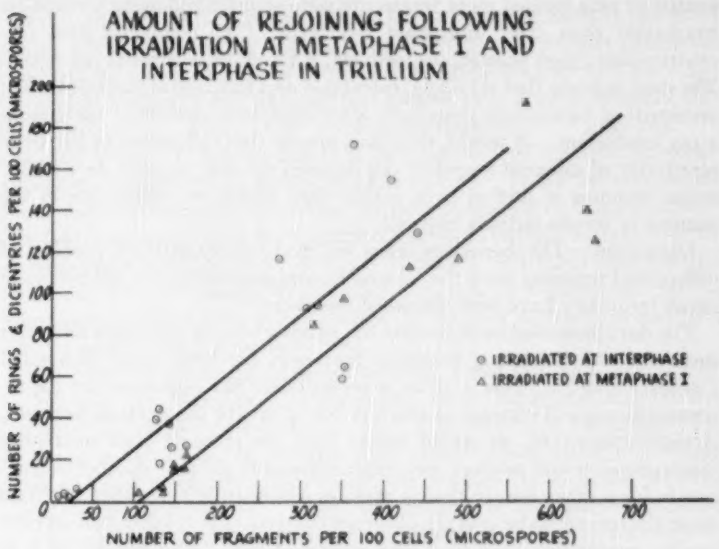

FIGURE 1

with the dicentrics. A few polycentric configurations were observed and these were included with the dicentrics. Tricentrics were counted as two dicentrics, etc.

Using the data given in tables 1 and 2, straight lines were fitted by the method of least squares to the regression of the number of rejoins (dicentrics plus rings) on the number of fragments (Fig. 1). These lines ap-

TABLE 3

SUMMARY OF DATA FROM TABLES 1 AND 2

STAGE IRRADIATED	NO. OF ANAPHASES ANALYZED	NO. OF FRAGMENTS (F)	NO. OF DICENTRICS (D)	NO. OF RINGS (R)	$\frac{D}{R}$	$\frac{F}{D}$	$\frac{F}{R}$	$\frac{F}{D+R}$
Metaphase	1161	2548	323	93	3.5	7.9	27.4	6.1
Interphase	3455	3411	652	246	2.7	5.2	13.9	3.8

pear to be very nearly parallel. By means of an analysis of variance the observed deviation from parallelism was found not to be significant. The average regression is significant, and the position or means were also found to differ significantly.

The data from tables 1 and 2 are summarized in table 3. Irradiation at metaphase yielded 3.5 dicentrics for every ring as compared with 2.7

following irradiation at interphase. In the last three columns the ratio of dicentrics and rings to fragments is given for each stage. For each dicentric or ring formed more fragments were counted following metaphase irradiation than after interphase treatment. The combined data (dicentrics plus rings) showed, respectively, 6.1 and 3.8 fragments per rejoin. The data indicate that rejoining, calculated as a function of fragmentation, occurred 1.6 times more frequently after interphase than after metaphase x-ray irradiation. It would, therefore, appear that differences in apparent sensitivity of different stages of cell division are due, in part, to a differential rejoining of broken ends, rather than wholly to a difference in the number of breaks initially induced.

Discussion.—The discussion below will deal mainly with the problem of differential rejoining since the various factors responsible for differences in break frequency have been discussed elsewhere.[5]

The data presented indicate that the number of both dicentrics and rings increases with increasing fragment frequency for both stages irradiated (tables 1 and 2). This is to be expected since the opportunities for rearrangements will increase as the number of breaks per nucleus increases. Actually, therefore, we would expect that the stage of high sensitivity (metaphase) would produce *more* rearrangements per unit number of fragments for a given dosage than a stage of low sensitivity. Since the data show the reverse to be true the observed differences in rejoining are of even greater significance than they may appear at first. This conclusion is in agreement with Bishop's[6] suggestion that peak sensitivity is related to a prevention of restitution but in disagreement with Catcheside's[6] conclusion "that the speeds of reunion and therefore of restitution do not alter much over a great part of the nuclear cycle, and that the internal controlling conditions are relatively constant" (p. 286).

Our results do agree in principle with the results of Bozeman and Metz[7] that the percentage of rearrangements observed in F_1 larvae of irradiated Sciara oöcytes is dependent upon the stage irradiated. They found different types of rearrangements to reach maxima at different stages of division. Adjacent repeats were highest at prophase and inversions were highest at anaphase. Deletions were high at prophase and metaphase and much lower at anaphase.

Changes in the definitive number of aberrations observed following a given dose of radiation at different stages may be due to (1) a difference in the initial number of breaks (or potential breaks) induced or to (2) a difference in the amount of rejoining of open breaks or (3) to a combination of both. The data presented show that following irradiation of different stages a difference in the amount of rejoining does occur but that it is not sufficient to account for the observed change in sensitivity based on fragment counts. It may, therefore, be concluded that a difference in the

number of initially produced breaks (or potential breaks) must also be concerned in determining the ultimate yield of breaks.

Various estimates have been made of the percentage of initially produced breaks which rejoin in the original sequence.[8-11] The present data indicate that the amount and kind of detectable rejoining varies with the stage of division irradiated and suggests that undetectable rejoins probably also vary with stage. Since other factors (temperature,[12-14] infra-red[15, 18] and ultra-violet[16, 17]) are also known to have an effect on rejoining, it would appear that rejoining is a highly variable process. To further complicate the situation rejoining is considered to be non-random in Tradescantia.[19] It would thus appear that the relationship between initial breakage and the amount and kind of rejoining is highly variable. It follows that sizeable errors might be introduced by carrying over data on a particular type of aberration obtained from one organism (or stage of division) to another. Unfortunately this is sometimes done.

The inverse relationship between breakage sensitivity and tendency of broken ends to rejoin may be of significance in the study of changes in the frequency of certain types of aberrations. For instance, if the capacity for rejoining is automatically reduced when many breaks occur in a single nucleus, a high break frequency would not produce the expected percentage of complex aberrations. This would account for the observed decrease in 2-hit aberrations at higher dosages,[20] as well as reduced percentage of dicentrics and rings observed (see above) at the most sensitive stages in Trillium.

In the presence of a low frequency of rejoining we would expect an increase in the percentage of acentric fragments or deletions and hence greater lethality. Therefore, aberrations recovered in the offspring of irradiated organisms would not necessarily represent a reliable picture of total chromosome breakage since cells irradiated in the most sensitive stages would be the least likely to produce viable gametes or progeny. It seems likely, therefore, that a sensitivity curve based solely on recoverable rings, translocations, inversions, etc., would differ from a curve based on the terminal and interstitial deletions (cf. 7, 21). Actually a curve based on the latter would probably be a more reliable indication of expected mortality since aberrations involving rejoins would be, as a whole, much less lethal than the loss of a portion of a chromosome.

Since mutations have been presumed by a number of investigators to be associated with primary breaks, it would be interesting to know whether gene mutation rate also varies widely with the stage of division irradiated. So far this aspect of mutation has not been sufficiently well investigated. It has been shown that x-ray induced mutation rate is higher at first metaphase than at early prophase in Prunus and Oenothera[22] and that it varies considerably at different stages of microsporogenesis in Tradescantia.[24, 25]

It is also higher in young than in mature pollen grains of *Antirrhinum majus*.[12] It thus seems plausible that mutation rate will be found generally to vary with the stage irradiated. It should therefore be realized that estimates of gene size based on irradiation of a particular stage (e.g., mature sperm of Drosophila) do not necessarily represent the true size of a "working" gene. If the changes in mutation sensitivity are as great as those of break sensitivity, estimates of gene size may well be off by a factor of several hundred or even several thousand per cent. In this connection, it is of interest to note that sensitivity volume of a virus actually does change during various stages of growth.[13]

In the present study the two stages investigated are known to differ with respect to the following factors: matrix, nuclear membrane, proximity of different chromosomes or chromosome parts, spiralization and stress of spiralization, degree of polarization, amount of attraction or repulsion, cytochemical constitution and physiological activities. The relative importance of these various factors in determining the behavior of chromosomes following irradiation must await further investigation.

Summary.—Differential rejoining has been studied in microspores of *Trillium erectum* following x-irradiation at stages of high and low apparent sensitivity to breakage. The stages irradiated were meiotic first metaphase and early post-meiotic interphase. Fragments, dicentrics and rings were scored at microspore anaphase. The numbers of dicentrics and rings observed were used as a measure of rejoining. The data indicate a significantly higher amount of rejoining in cells irradiated at early interphase than in those irradiated at meiotic metaphase. However, the increase in rejoining is not sufficient to account for the observed decrease in fragmentation.

It is therefore concluded that the observed difference in sensitivity of the two stages studied is due in part to a difference in number of primary breaks (or potential breaks) and in part to a difference in the amount of rejoining which subsequently occurs. In the material studied a high sensitivity to primary breakage is associated with a low frequency of rejoining and vice versa. Certain implications of these conclusions on calculations of mutation rate and gene size are discussed.

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*THE CONSTANCY OF DESOXYRIBOSE NUCLEIC ACID IN PLANT NUCLEI**

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For a number of years considerable interest has been centered in the role of nucleic acids in cellular processes. Recently desoxyribose nucleic acid (DNA) has been shown to possess interesting characteristics that have led several workers to consider it an essential component of the gene.[1–2] DNA is probably a universal constituent of plant and animal nuclei. Its low turnover rate to radioactive phosphorus and nitrogen, in non-dividing tissues, is evidence for a chemical stability considerably greater than that of other cell components.[4, 5] Moreover, recent analytic data on the actual amounts of DNA within nuclei have suggested that it possesses a quantitative stability as well.

Computations on the amount of DNA per nucleus have been made in two ways. Chemical analyses of large numbers of cells, with the number present estimated by sample counts, have given the average amount of

DNA, per nucleus, for many thousand cells. Light absorption measurements made through a microscope have, on the other hand, yielded data on individual nuclei. Both methods involve technical difficulties and are open to certain criticisms. Nevertheless both biochemical[1, 2, 6] and microscopic[3, 7, 8] measurements by a number of different investigators have supported the concept first proposed by Boivin, Vendrely and Vendrely[1] that within the tissues of an organism the actual amount of DNA per nucleus is apparently constant.

Boivin, *et al.*, have considered that all somatic cells of an animal possess the same amount of DNA, with the gametes containing half this value. A number of discrepancies from this simple relation have been reported. Although some of these may be attributable to errors in the analytical techniques employed, at least two processes obviously involve naturally occurring variations. Ris and Mirsky[7] first showed that DNA classes with the ratio 1:2:4 occur in the rat liver associated with polyploid nuclei. It was later found[8] that such DNA classes, apparently associated with polyploidy and polyteny, occur in a variety of animal tissues. Variation of another type occurs in association with the mitotic cycle. Since DNA in several animal tissues has been found to increase in interphase preceding cell division to twice the diploid amount, interphase nuclei of dividing tissues may contain anywhere from two to four times the amount found in the haploid sperm.[3] These variations in the amounts of DNA, associated with mitosis or DNA classes, cannot be considered in disagreement with the basic tenets of Boivin's theory. It is clear that, at least in many different animal tissues, the amount of DNA per nucleus is under rather definite quantitative restrictions. Interphase nuclei of non-dividing tissues, with a few possible exceptions, have been found to contain an amount of DNA approximately 2, 4, 8, etc., times that found in the gametes. A haploid amount of DNA has been found in sperm,[1, 2] spermatids[3, 9, 10] and male and female pronuclei.[5] In general these estimates of the nucleic acids in cells are at present accurate to 10 or 20%. The question of how precisely the quantitative relations are obeyed must await more accurate techniques.

The quantitative behavior of DNA in plant tissues has as yet received little attention. Schrader and Leuchtenberger[11] have recently shown that the amount of DNA varies from tissue to tissue in Tradescantia. The data presented, although they clearly show that the amount of DNA is different in different tissues, are not extensive enough to demonstrate the type of variability. Does DNA tend to occur in the well-marked constant units found for a number of animal tissues, or, as might be inferred from the work of Schrader and Leuchtenberger, does it fail to follow any definite quantitative pattern? The present work was primarily designed to answer this question.

Material and Methods.—Two plant species have been studied in detail and two others briefly. Plants of *Tradescantia paludosa* were kindly provided by Dr. J. M. Beal of the University of Chicago. Corn plants (*Zea mays*) were obtained from several sources, particularly commercial Golden Bantam, and three strains from the collection of Dr. M. M. Rhoades of the University of Illinois. Plants of *T. canaliculata* were collected in the Chicago region, and cultivated plants of *T. "virginiana,"* differing slightly from the typical native form, came from a Chicago garden. All material was fixed in neutral 50% formalin (one part of the stock 40% formaldehyde solution to one part of distilled water, with calcium carbonate added) immediately after removal from the plant. Pieces were small, with the smallest dimension rarely exceeding 1 mm., and buds were opened to permit rapid pentration. Fixation was for at least three hours. Material was thoroughly washed, sectioned in paraffin, and stained with the Feulgen reagent for one hour after an hydrolysis in normal hydrochloric acid of 14 minutes at 60°C. In the present study, wherever possible, all tissues to be compared were mounted together on the same slide, and where this was not done, a section of tissue previously studied was mounted beside the unknown. Control sections in five of the eight slide series studied gave essentially similar values. In the remaining three the Feulgen intensity of the control sections were slightly below that usually obtained, and all values from these series were consequently raised.

The amount of Feulgen dye in individual nuclei was estimated by photometric determinations made through a microscope. The technique was approximately as described previously[3, 12] with the following exceptions: Essentially monochromatic light was isolated by a Beckman spectrophotometer with a slit width of 0.1 or 0.03 mm. Measurements were made with a Leitz achromatic-aplanatic condenser, N.A. 1.4, a 90 × Leitz 2-mm. oil immersion apochromatic objective, N.A. 1.32, and a 20 × Bausch and Lomb coated hyperplane ocular, containing an iris diaphragm to minimize distortion from internal reflection. The microscope image, enlarged 1000 times, was projected on a field diaphragm, which allowed an area 2, 3, 4 or 5 mm. in diameter, taken in the center of the nuclear image to fall on the phototube. Measurements were made with a battery-powered 1P21 electron multiplier phototube, with output leads connected to a Farrand type B control unit and a Rubicon galvanometer. Measurements of corn tissues were made at the absorption peak of the Feulgen dye, 560 mμ. Tradescantia nuclei at this wave-length were too dark to measure accurately, and consequently were measured considerably off the maximum absorption, at 615 mμ. The extinction at this wave-length was about 22% of that at 560 mμ.

Photometric determinations of biological material are subject to a variety of technical variables.[3] The most important of these in the present work were probably caused by the irregular distribution of the stainable com-

ponents in the nucleus and inaccuracies in estimating nuclear volume. A strong formalin fixative, where rapid penetration is aided by using small pieces of tissue, tends to keep the chromatin in its natural extended state. Where the nuclei measured were markedly irregular, e.g., in meiotic diakinesis, measured values tended to be low. The apparatus was calibrated

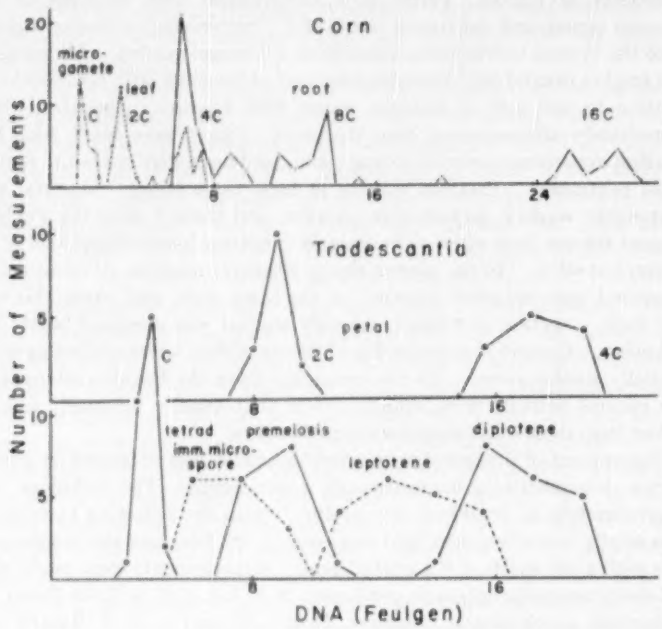

FIGURE 1

Distribution of DNA (Feulgen) measurements on individual nuclei of corn and Tradescantia tissues. The amount of DNA is shown in arbitrary units. *Upper graph:* Corn microgamete nuclei from pollen grains (dashed lines), leaf nuclei (dotted lines) and nuclei from corn root zone of elongation (solid lines). *Middle graph:* Tradescantia petal nuclei from mature flower. *Lower graph:* Stages in Tradescantia pollen formation from developing anthers, showing measurements on premeiosis (preleptotene), leptotene, diplotene, tetrad and immature microspore stages.

as previously, and the non-specific light loss was found to be negligible by measuring unhydrolyzed controls.

The advisability of using the Feulgen reaction for quantitative microphotometric determinations of DNA has been discussed by a number of workers. It is now generally agreed that, where staining procedures are properly carried out, the Feulgen reaction can give an accurate relative

estimate of the DNA in nuclei.[3, 7, 10] Since the actual intensity of the dye produced can be markedly altered by such factors as the type of fixative used, size of the tissue fixed and slight changes in hydrolysis conditions no attempt has been made here to convert the data presented into absolute amounts of DNA. All values are given in the arbitrary units used elsewhere.[3, 8] The measured extinction (E) of a central region 2 to 5 microns in diameter of an uncut nucleus has been multiplied by the squared radius of the measured area (C^2) and divided by the fraction (F) of the total nuclear volume included in the measured region. Units $= \frac{EC^2}{F}$, where $F = \frac{R^3 - (R^2 - C^2)^{3/2}}{R^3}$, and R is the radius of the nucleus. Markedly aspherical nuclei were not measured. Where nuclei were slightly ellipsoid, R was taken as the mean of major and minor axes.

Results.—(*A*) *Non-Dividing Tissues:* Photometric measurements made on tissues where mitoses were uncommon tended to fall in certain well-marked classes. Means of these classes fit in the series 1:2:4:8:16:32. The distributions of measured values from corn leaf and root, and Tradescantia petal are shown in figure 1. Means for all measurements are given in tables 1 and 2. The values are expressed as the total number of dye molecules per nucleus, in arbitrary units, and thus constitute a relative estimate of the DNA in nuclei. In similar tissues the arbitrary units are about 2.5 times higher for Tradescantia than for corn. Since the Tradescantia tissues were measured at a wave-length giving only about 22% of maximum extinction, these nuclei contain approximately 10 times the DNA found in corn.

The lowest values for Tradescantia have been found in the young microspore nuclei (tetrad stage), young generative nuclei and tube nuclei; and for corn in the microgamete nuclei of mature pollen, all presumably haploid. Most nuclei in both species were found to have twice ($2C$) or four times ($4C$) the haploid amount. Nuclei falling in class $8C$ have been found in Tradescantia stamen hairs, corn root and root cap and in the scutellum nuclei of the corn kernel. In the root and scutellum $16C$ nuclei also occur. In a few tissues, i.e., the root cap and zone of elongation in corn, and in the mature stamen hairs in Tradescantia, class $2C$ nuclei are rare and almost all nuclei belong to the higher classes. In young stamen hairs, however, class $2C$ cells are common. Measurements on the aleurone and endosperm of the corn kernel, tissues long known to be triploid through the joining of one microgamete with the $2N$ endosperm nucleus, fell in the series 3:6:12:24. Most aleurone nuclei in the kernels studied fell in class $6C$. Endosperm nuclei were measured in young ears, since those of the mature kernel are highly irregular. The smaller classes tended to be peripheral.

In the corn root zone of elongation most nuclei fell in classes 4*C* and 8*C*. However, in certain rows of cells forming the major vessels of the root, larger classes (up to 32*C*) were found. When all the nuclei in such a vessel were measured in order from the root tip back to about 1500 μ from the tip,

TABLE 1

AVERAGE AMOUNTS OF DNA (FEULGEN) PER NUCLEUS IN VARIOUS TISSUES OF CORN (*Zea mays*)

CELL TYPE	DNA CLASS	DNA IN ARBITRARY UNITS	STANDARD ERROR	NO. MEASURED
Microgamete nucleus (interphase)	*C*	1.6*	0.06	22
Leaf				
(interphase)	2*C*	3.4*	0.05	23
	2*C* → 4*C*	4.8*	..	1
	4*C*	6.6*	0.12	19
(prophase)	4*C*	6.9	0.12	10
(telophase)	2*C*	3.2	0.09	12
Root cap (interphase)	4*C*	6.9	0.12	15
	8*C*	12.6	0.33	22
Root elongation zone (interphase)	4*C*	6.6*	0.06	29
	4*C* → 8*C*	8.7*	..	6
	8*C*	12.5*	0.27	19
	8*C* → 16*C*	20.2*	..	2
	16*C*	26.1*	0.45	17
	16*C* → 32*C*	33.8	..	2
	32*C*	49.0	..	6
Root meristem				
(interphase)	2*C* → 4*C*	5.4*	0.21	36
(prophase)	4*C*	6.4*	0.09	15
(telophase)	2*C*	3.2*	0.05	20
Embryo (interphase)	2*C*	3.6	0.07	15
	4*C*	7.1	0.13	15
Scutellum (interphase)	2*C*	3.3	0.09	20
	4*C*	6.4	0.11	40
	8*C*	12.6	0.24	15
	16*C*	26.2	..	5
Aleurone (interphase)	3*C*	4.8	0.07	15
	6*C*	10.1	0.20	40
	12*C*	20.5	0.60	15
Endosperm (interphase)	3*C*	5.0	0.06	15
	6*C*	9.3	0.14	17
	12*C*	19.1	0.60	16
	24*C*	38.0	1.20	10

* Data graphed in figure 1 or figure 2.

all classes from 4*C* to 16 or 32*C* were usually represented, in ascending order. From 6 to 12 nuclei have been found together from each class, and between these groups anywhere from 0 to 6 intermediate values have been obtained. It is thus likely that these large vessel cells, while remaining in interphase undergo a periodic DNA doubling. Values from a few such series are com-

bined in figure 1 and table 1; in table 1 only values falling outside the expected interclass variability have been considered as intermediate. More work on this process is in progress.

Only one intermediate value has been obtained from leaf tissue in more than 150 measurements, and this came from the leaf base where a few mitoses were present. In the differentiated leaf, where cell division is absent, no intermediates have been found. It seems probable that intermediate amounts occur only when cells are synthesizing DNA for cell division (see below) or periodic DNA doubling.

(*B*) *Dividing Tissues:* The quantitative changes in DNA during the mitotic cycle were studied in corn root and leaf meristem, and in the root

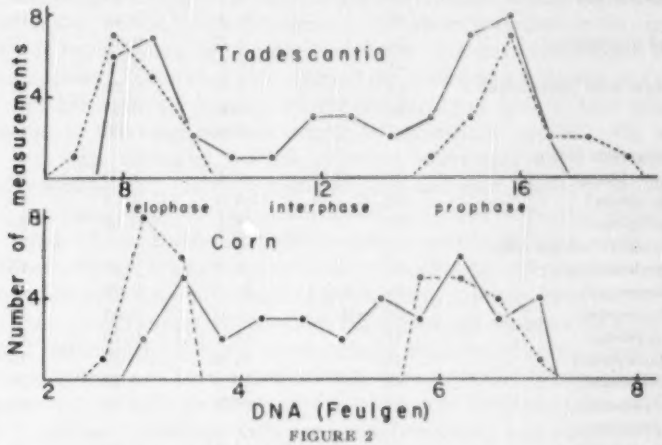

FIGURE 2

Distribution of DNA (Feulgen) measurements on individual nuclei from the root meristem of corn and Tradescantia. The amount of DNA is shown in arbitrary units. Interphase nuclei (solid lines), prophase nuclei (dotted lines at right), and telophase nuclei (dotted lines at left).

meristem and sporogenous tissue of Tradescantia. In all these tissues the process was essentially the same, paralleling that described previously for animals.[3] Where mitotic figures were common, measurements on interphase nuclei scattered widely between classes 2*C* and 4*C* (Fig. 2). Prophase values fell in 4*C* and telophases in 2*C*. Late prophase, metaphase and anaphase cells were too irregular to measure, but the behavior of DNA in these stages can easily be inferred. Apparently DNA increases during interphase to double the common diploid amount, reaching the 4*C* value at or before the visible beginning of prophase. During prophase and metaphase probably no DNA is synthesized. The 4*C* amount is then cut in

two at anaphase, and the telophase nuclei each possess the 2*C* value. In the tissues studied no 8*C* or 16*C* prophase stages have been found, although

TABLE 2

AVERAGE AMOUNTS OF DNA (FEULGEN) PER NUCLEUS IN VARIOUS TISSUES OF *Tradescantia paludosa*

CELL TYPE	DNA CLASS	DNA IN ARBITRARY UNITS	STANDARD ERROR	NO. MEASURED
Leaf (interphase)	2*C*	8.5	0.07	20
Root meristem				
(interphase)	2*C* → 4*C*	13.1[a]	0.47	30
(prophase)	4*C*	16.2[a]	0.29	15
(telophase)	2*C*	8.3[a]	0.16	15
Tapetum (interphase)	2*C*	8.5	0.10	33
	4*C*	16.4	0.36	14
Petal (interphase)	2*C*	8.6[a]	0.13	15
	4*C*	16.9[a]	0.27	15
Stamen hairs (interphase)	2*C*	8.5	0.26	10
	4*C*	16.6	0.26	21
	8*C*	33.6	0.65	15
Sporogenous tissue				
(interphase)	2*C* → 4*C*	13.1	1.00	15
(prophase)	4*C*	16.0	0.24	15
(telophase)	2*C*	8.1	0.19	15
Microspore mother cells				
(preleptotene)	2*C*	8.7[a]	0.20	15
(leptotene)	2*C* → 4*C*	12.6[a]	0.39	15
(leptotene)	2*C* → 4*C*	13.0	0.54	20
(zygotene)	4*C*	16.1	0.20	20
(pachytene)	4*C*	16.8	0.39	15
(diplotene)	4*C*	16.9[a]	0.28	20
(diakinesis)	4*C*	16.3	0.25	20
Microspores				
(tetrad stage)	*C*	4.4[a]	0.04	30
(early interphase)	*C*	4.0	0.10	20
(late interphase)	*C* → 2*C*	5.9	0.34	10
(late interphase)	*C* → 2*C*	7.4[a]	0.24	25
(prophase)	2*C*	9.2	0.20	15
Pollen tube nuclei				
(early interphase)	*C*	4.2	0.09	15
(late interphase)	*C*	4.1	0.07	27
Generative nuclei				
(early interphase)	*C*	4.3	0.11	14
(late interphase)	2*C*	8.5	0.08	16

[a] Data graphed in figure 1 or figure 2.

their occurrence might be expected in connection with the division of 4*C* or 8*C* nuclei. In the corn root zone of elongation where most interphase nuclei fall in class 4*C*, cells apparently proceed directly into a 4*C* prophase without DNA synthesis, the 4*C* level then being restored in the following

interphase. In the stamen hairs of Tradescantia most cell division was found to take place in developing buds, where 2*C* nuclei were common, following the usual pattern. No division stages were seen in the 4*C* or 8*C* nuclei from mature flowers.

(*C*) *Meiosis:* The behavior of DNA during pollen formation was studied only in Tradescantia, since the chromosomal material during most meiotic stages of corn was found too irregular for measurement. Anthers of Tradescantia, when first differentiated, are filled with rapidly dividing sporogenous cells. Measurements of these cells, in young anthers, tended to follow the pattern found in other dividing tissues (table 2). Prophases fell in 4*C*, telophases in 2*C*, and interphases were scattered in between. The beginning of meiosis is marked by general cessation of mitotic activity in the anther, except at the periphery. The nuclei measured in this preleptotene resting stage, before the thread-like chromosome structure becomes apparent, fell in class 2*C*. During the subsequent leptotene, microspore mother cells were found to increase in amount of DNA so that measurements on this stage were intermediate between 2*C* and 4*C* (Fig. 1). The next stage measured was late zygotene, where only a few unpaired strands were visible. In these cells the DNA had approximately doubled (4*C*), and throughout the rest of the meiotic prophase no further increase was found. Immediately after the second maturation division, while still in the tetrad stage, the microspore nuclei fell in class *C*. Measurements on microspores from three later stages of development indicate that a comparatively long period ensues during which the microspore contains the class *C* amount, followed by a fairly rapid increase prior to mitosis. Data for microspores from one anther, intermediate between *C* and 2*C*, are graphed in figure 1. At early prophase of the microspore division nuclei fall in class 2*C* and are divided into tube and generative nuclei, each with the class *C* amount. Some time before anthesis the generative nucleus increases to 2*C*, but the tube nucleus remains at the *C* amount. In the mature pollen most generative nuclei become very elongate and are thus impossible to measure, but a few continue to be spheroidal, and in these the DNA can be determined. The two microgamete nuclei resulting from division of the generative nucleus were not measured in Tradescantia, but the microgamete nuclei were studied in corn and fell in class *C* (Fig. 1 and table 1).

The course of DNA in Tradescantia meiosis can thus be outlined briefly as follows: The earliest microspore mother cells fall in class 2*C*, increasing during leptotene and possibly also during zygotene to 4*C* for the remaining stages of the meiotic prophase. The four tetrad nuclei resulting from the maturation divisions each have the *C* amount, increasing to 2*C* before the microspore mitosis. This division results in class *C* tube and generative nuclei, the latter increasing to 2*C* before anthesis. The generative nucleus apparently divides to form two haploid (*C*) microgamete nuclei.

(*D*) *Strain and Species Differences:* As mentioned above, nuclei of *Tradescantia paludosa* contain about ten times the DNA found in corn nuclei of the same class. The amount of DNA in *T. paludosa* leaf nuclei was compared with that found in two other closely related Tradescantia species (table 3). Both species have a haploid chromosome number of 12, twice that of *T. paludosa*, and thus it is not surprising to find the diploid nuclei contain about twice the DNA. Interspecific variation in amounts of DNA has been reported for animals and is not unexpected in view of the deletions, duplications, polysomaty, etc., considered to accompany evolution. On the same basis one would also expect the amount of DNA per nucleus might differ to some extent in various strains of the same species. To test this possibility similar tissues from two corn strains, differing in amount of heterochromatin, were mounted together and measured. Strain *A* (table 3) contained several *B* chromosomes and knobs, and the interphase nuclei showed the chromocenters associated with them.[13] Strain *B* had no *B* chromosomes and contained only a small amount of

TABLE 3

AVERAGE AMOUNTS OF DNA (FEULGEN) PER NUCLEUS IN TISSUES OF TWO STRAINS OF CORN AND THREE SPECIES OF TRADESCANTIA

	LEAF			ROOT		
	DNA IN ARBITRARY UNITS	STANDARD ERROR	NO. MEASURED	DNA IN ARBITRARY UNITS	STANDARD ERROR	NO. MEASURED
Corn strain A	6.4	0.12	15	6.6	0.08	15
Corn strain B	7.2	0.10	15	7.4	0.12	15
T. paludosa	8.5	0.08	25			
T. "virginiana"	16.8	0.20	25			
T. canaliculata	16.1	0.18	25			

heterochromatin on chromosome 6. Class 4*C* nuclei, from root and leaf, showed a difference in DNA between strains of about 10%.

Discussion.—The data presented indicate that DNA follows quantitative restrictions of the same general type reported for animal tissues. Three points may be stressed: (1) The amount per nucleus shows a marked step-like occurrence. (2) There is a duplication with mitosis and a reduction with meiosis. (3) Since species and strains have characteristic amounts of DNA it is apparent that the quantities involved are directly associated with the genotype. At least for the present these factors seem best interpreted by considering DNA as a component of the gene.

A considerable amount of recent cytological evidence has accumulated that points to the occurrence of "supernumerary chromonemal reproductions" as they have been called by Lorz[14] in numerous plant tissues. In several instances chromosomes have been described as 2, 4 or 8 stranded (polytene).[15,16] Endomitotic cycles, such as those described in the tapetum of Spinacia[17] or tomato,[18] are known to cause doubling of the chromo-

some number, and where differentiated resting nuclei have been stimulated to divide by auxins[19] or other treatment, polyploid nuclei with 2, 4, 8, etc., times the diploid chromosome number have been found. As pointed out by Schrader and Leuchtenberger it seems likely that the occurrence of varying amounts of DNA is associated with such factors.

The nuclei measured in the course of the present work naturally represent an extremely small sample. Nevertheless it is interesting that so few intermediate values have been found in non-dividing tissues. This would seem to indicate that the "endomitotic" processes, during which DNA doubling occurs, are comparatively rapid, and that unsynchronized chromonemal reproductions of the type seen in Rhoeo[19] are rare in the tissues studied. They may be more common in the older nuclei, which are often too irregular to measure.

The conclusion seems unavoidable, from both cytological and photometric evidence, that many, and in some tissues most, cells typically contain multiple chromonemal sets. The role played by these cells in the economy of the organism can at present only be conjectured. By analogy with the situation in autopolyploid plants one might expect the physiological balance to be altered. It has often been suggested that endomitotic gene doubling is associated with differentiation.[20, 21] However, in Tradescantia stamen hairs, as well as in some mammalian tissues, the higher classes do not appear until differentiation is completed.

Summary.—Photometric determinations on individual Feulgen-stained corn and Tradescantia nuclei support the view that DNA occurs in well-marked units characteristic of the strain or species. Nuclei with 2, 4, 8, 16 or 32 times the haploid (microgamete) value occur. Preceding mitosis DNA increases in interphase to twice the dipoloid amount. In meiosis the DNA is reduced, so that the microgamete contains half the diploid value.

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*ALLELISM OF SECOND CHROMOSOME LETHALS IN D. MELANOGASTER**

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Our knowledge of the genetic composition of populations, other than that of human blood group genes, is based primarily on lethal chromosomes from populations of Drosophila. Paradoxically, because of the advanced genetic techniques in Drosophila, our information for species of this genus consists of *chromosomal* frequencies although the dynamics of population genetics depends upon *gene* frequencies. It would be possible to analyze Drosophila populations for specific gene loci but it has proved more profitable to evaluate the easily collected lethal chromosome data by estimating the number of loci on a given chromosome at which lethal alleles may exist. This estimation, which can be made by determining the frequency of allelism between lethals of independent origin, has been made by Wright[1, 2] for the third chromosome of *D. pseudoobscura* (285–289 loci) and by Ives[3] for the second chromosome of *D. melanogaster* (495 loci). In connection with experimental populations which are exposed to continuous gamma irradiation, it has been necessary to determine the number of loci which are capable of mutating to lethality under the influence of these radiations.

Flies of the Oregon-R strain of *D. melanogaster* carrying lethal-free second chromosomes were placed in a population cage and were allowed to oviposit on food in small plastic cups throughout the day (8 hrs.) or overnight (16 hrs.). (See Wallace[4] for a detailed description of the cages and the cups.) At the end of each egg-collecting period, a fresh cup was exposed to the parental flies, and the old cup, with its eggs, was placed in a cage which encircled a 500-mg. radium bomb. To keep the developing flies of each cup separate from the rest, a thin-walled plastic tube was inserted into the food of each cup and was plugged at its free end with cotton.

The flies developing under these conditions were exposed throughout their developmental stages and for part of their adult life to a constant dose of gamma rays of approximately 5 r per hour. After 18 days (426 hrs. or 2175 r, average), males were removed and mated individually in vials with CyL/Pm females. (The CyL chromosome carries the dominant genes Curley and Lobe and two inversions which suppress most of the crossing-over.) Several CyL/+ F1 males of each culture were mated singly in vials with CyL/Pm females, and the CyL/+ F2 males and females of each culture were inbred in vials to determine the lethal mutation rate. If a lethal had been induced on a treated chromosome, this was noticed by the absence of wild-type flies in the F3. Each suspected lethal was confirmed by mating for an additional generation in a regular culture bottle. After confirmation, 125 lethals which had arisen in different original males and, therefore, were independent in origin, were subcultured in four bottles in order to obtain flies for the allelism tests. With the exception of the latter tests which were kept at room temperature (22° ±) all phases of the experiment were carried out at 25°C.

To determine the frequency of induced mutations, 3772 second chromosomes derived from 336 treated males were analyzed; 456 were lethal. The frequency of lethal chromosomes, then was 12.09 ± 0.53%. The frequency of lethal genes, calculated by means of the Poisson distribution, was 12.89 ± 0.60%.

The tests for the frequency of allelism were made by intercrossing CyL/+ flies from 100 lethal cultures of independent origin. (The remainder of the 125 cultures originally chosen either produced too few flies for the required matings or gave rise to small numbers of wild-type flies which could have obscured the test.) Fourteen of the 4950 $\left(\frac{100 \times 99}{2}\right)$ matings failed to produce wild-type flies. The probability, then, that one tested chromosome was allelic to another is 0.28%. (The limits of the 95% confidence interval of this frequency are 0.16% and 0.48%).

The minimum number of loci, n, capable of mutating to lethality is the inverse of the probability that one lethal gene is allelic to another. This number can be calculated from the frequency of lethal chromosomes (a = 12.09 ± 0.53%), the frequency of lethal genes (b = 12.89 ± 0.60%) and the frequency of allelism between lethal chromosomes (c = 0.28% with limits 0.16% and 0.48%): $n = b^2/ca^2$. The most probable minimum number calculated from our data is 400. By substituting the most divergent values of a, b and c into the equation, the limits of the minimum number may be estimated as 234 and 718.

The distribution of allelic lethals among the 100 chromosomes which were tested gives a quasi-independent confirmation of the above estimate of the minimum number of loci. In one case chromosome A was allelic to both

chromosomes *B* and *C*, but *B* and *C* were non-allelic, therefore, it is known that the tests for allelism involved lethal genes at 101 loci. Among these 101 lethals there were 73 which occurred only once and 14 which occurred twice. For any given number of potentially lethal loci it is possible to predict the distribution of singles, pairs and triplets among 101 independently chosen lethals by means of the Poisson series. If $n = 234$, there should be 65.6 singles, 14.2 pairs and 2.0 triplets; if $n = 400$, these values should be 78.4, 9.9 and 0.8; if $n = 718$, 87.7, 6.2 and 0.3. The expected distribution when $n = 718$ is significantly different from the observed distribution; it is probably that the minimum number of lethal loci lies nearer the calculated 400.

It is of interest to note the similarity between the number of loci capable of mutating to lethality under the influence of gamma rays (400 with limits of 234 and 718) and the number capable of mutating spontaneously (495 with limits of 285 and 705). This agreement indicates that these loci are so situated that an ionizing particle is likely to affect only one at a time. Since nearly 2000 bands have been recorded in the second chromosome of *D. melanogaster* in salivary preparations and since induced lethals have frequently proved to be small deficiencies (Slizynski[8]), it seems likely that loci mutating frequently to lethality may be separated from one another by material relatively inert in this respect.

In conclusion, several points should be emphasized. The 400 loci calculated above represent a minimum number of loci which can mutate to lethality. The maximum number could be substantially larger but, nevertheless, remain undetected because of great differences in the mutation rates of different loci. It should be noted, too, that observed allelism does not prove identity; overlapping deficiencies may act as allelic lethals. These considerations have been pointed out by Wright[1] in his analysis of Dobzhansky's data.

Summary.—Through an analysis of the frequency of allelism of 100 second chromosome lethals induced in *D. melanogaster* by chronic gamma-ray treatment, it has been estimated that the minimum number of loci capable of mutating to lethality under these conditions is 400 (234–718).

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COHOMOLOGY THEORY OF ABELIAN GROUPS AND HOMOTOPY THEORY II

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1. Generic Homology Groups.—The cohomology groups of a group Π are the groups of the cell complex $K(\Pi, 1)$ which has as its cells in each dimension $q \geqq 1$ the q-tuples $[x_1, \ldots, x_q]$ of elements $x_i \in \Pi$, with $\partial[x] = 0$ and

$$\partial[x_1, \ldots, x_q] = [x_2, \ldots, x_q] + \sum_{i=1}^{q-1} (-1)^i [x_1, \ldots, x_i x_{i+1}, \ldots, x_q] + (-1)^q [x_1, \ldots, x_{q-1}]. \quad (1)$$

This boundary formula does not use inverses of the letters x_i, and no letter x_i is repeated in any one cell of the boundary. These two properties may be conveniently expressed in the complex $K(F, 1)$ constructed from the free group F with a denumerable set of free generators $g_1, g_2, \ldots$. Call an element of F *generic* if it is a product $x = 1 g_{i_1} g_{i_2} \ldots g_{i_k}$ of $k \geqq 0$ distinct generators, and call two generic elements x and y *disjoint* if they involve no generator in common. A cell $[x_1, \ldots, x_q]$ is generic if the entries $x_1, \ldots, x_q$ are generic and pairwise disjoint. Then formula (1) shows that the boundary of any generic cell is a linear combination (with integral coefficients) of generic cells. Consequently the generic cells span a subcomplex $K(F^*, 1)$ of $K(F, 1)$.

The usual proof[1] that the cohomology groups of a free group F are zero in all dimensions greater than 1 gives the integral homology groups of this "generic" complex $K(F^*, 1)$ as

$$H_q(K(F^*, 1)) = 0, \qquad q > 1 \quad (2)$$

$$H_1(K(F^*, 1)) = F_a, \quad (3)$$

where F_a is the free abelian group on a denumerable set of generators g_i,

$g_2, \ldots,$ and the isomorphism in (3) is given by the mapping $[x] \to \phi x$, where $\phi: F \to F_a$ maps each g_i into g_i.

The geometric applications (in particular Theorem 4 below) indicate that the cohomology groups appropriate to an abelian group Π will be those of a suitable cell complex which has the formal properties (2) and (3) relative to the generic elements of the free *abelian* group F_a. The first stage in the construction of such a complex $A(\Pi)$ will be the complex $A^0(\Pi) = K(\Pi, 1)$. The generic subcomplex $A^0(F_a^*)$ satisfies (3) but not (2). Indeed, if g and h are any two generators of F_a, then, because of the commutative law, $\partial[g, h] = [h] - [gh] + [g] = \partial[h, g]$. Thus

$$[g, h] - [h, g] \tag{4}$$

is a 2-dimensional generic cycle; it is not a boundary. Similarly, for any three distinct generators g, h and k,

$$[g, h, k] - [g, k, h] - [h, g, k] + [h, k, g] + [k, g, h] - [k, h, g] \tag{5}$$

is a generic 3-cycle.

A *shuffle* of the string of letters $y_1, \ldots, y_r$ through the letters $x_1, \ldots, x_q$ is any sequence $z_1, \ldots, z_{q+r}$ consisting of the $q + r$ letters x_i, y_j in some order such that the x's alone and the y's alone appear in their given order. We define an operation $*_0$ on cells of $A^0(\Pi)$ as

$$[x_1, \ldots, x_q] *_0 [y_1, \ldots, y_r] = \sum (-1)^\epsilon [z_1, \ldots, z_{q+r}], \tag{6}$$

the sum extending over all shuffles, with the sign of each shuffle determined by ϵ, the number of pairs of indices (i, j) for which x_i follows y_j in the shuffle. With this notation, the generic cycles (4) and (5) may be expressed as $[g]*_0[h]$ and $[g]*_0[h]*_0[k]$, respectively. In general, we have

THEOREM 1. *If F_a is the free abelian group on generators $g_1, g_2, \ldots$, then for each dimension $q \geqq 1$ the integral homology group $H_q(A^0(F_a^*))$ of the generic subcomplex is a free abelian group with the generators*

$$[g_{i_1}]*_0[g_{i_2}]*_0 \ldots *_0[g_{i_q}], \qquad i_1 < i_2 < \ldots < i_q. \tag{7}$$

2. The Bar Construction for Complexes.—In general, a *product of excess* $k \geqq 0$ in a complex L is a bilinear function $a*b$ on the chains a, b of L such that, if $d(a)$ denotes the dimension of a, and if $d_k(a) = d(a) + k$,

$$d(a*b) = d(a) + d(b) + k \quad \text{(i.e., } d_k(a*b) = d_i a + d_k b) \tag{8}$$

$$a*(b*c) = (a*b)\underline{*}c, \tag{9}$$

$$b*a = (-1)^\epsilon a*b, \qquad \epsilon = (d_i a)(d_k b), \tag{10}$$

$$\partial(a*b) = (\partial a)*(b) + (-1)^{d_k(a)} a*(\partial b). \tag{11}$$

In particular, (6) defines a product of excess zero in $A^0(\Pi)$, for Π abelian.

Let L be an abstract cell complex with a product $\#$ of excess $k-1$. Define a new complex $M = \mathfrak{B}(L)$ whose cells are all expressions

$$\sigma = [\alpha_1|\alpha_2|\ \dots\ |\alpha_p], \qquad \alpha_i \text{ cells of } L, \tag{12}$$

where $|$ is short for $|_k$ (a k fold bar). This cell has dimension

$$d(\sigma) = d(\alpha_1) + \dots + d(\alpha_p) + (p-1)k$$

(each bar counts as k dimensions), and has boundary

$$\partial\alpha = \sum_{i=1}^{p} (-1)^{\epsilon_i}\,[\alpha_1|\ \dots\ |\alpha_{i-1}|\partial\alpha_i|\alpha_{i+1}|\ \dots\ |\alpha_p] + \sum_{i=1}^{p-1}(-1)^{\epsilon_i}[\alpha_1|\ \dots\ |\ \alpha_{i-1}|\alpha_i \# \alpha_{i+1}|\alpha_{i+2}|\ \dots\ |\alpha_p] \tag{13}$$

where $\epsilon_i = d_k(\alpha_1) + \dots + d_k(\alpha_i)$. In formula (13), if $\partial\alpha_i$ or $\alpha_i \# \alpha_{i+1}$ is not a cell but a chain, the term is to be expanded by linearity. One proves that $\partial\partial = 0$. Now define a new product $*$ in $\mathfrak{B}(L)$. For $\tau = [\beta_1|\ \dots\ |\beta_q]$ and σ as in (12), set

$$\sigma * \tau = \sum(-1)^{\epsilon}[\gamma_1|\ \dots\ |\gamma_{p+q}]$$

with the sum taken over all shuffles $\gamma_1, \dots, \gamma_{p+q}$ of the letters $\beta_1, \dots, \beta_q$ through the letters $\alpha_1, \dots, \alpha_p$. The sign ϵ of any shuffle is the sum $\sum d_k(\alpha_i)d_k(\beta_j)$, taken over all pairs of indices i, j, such that β_j comes before α_i in the shuffle. This new product has excess k in $\mathfrak{B}(L)$, and the new complex $M = \mathfrak{B}(L)$ contains L (as cells (12) with $p = 1$). Write $\mathfrak{B}(L, \#) = (M, *)$.

A complex L' with a product $\#'$ of excess $k-1$ is said to be a *reduction* of the complex L with product $\#$ of excess $k-1$ if there is a chain equivalence $f: L \to L'$ which maps L onto L' and which preserves products; i.e., is such that

$$f(a \# b) = (fa) \#' (fb) \tag{14}$$

for all chains a, b of L.

THEOREM 2. *If $(L', \#')$ is a reduction of $(L, \#)$, then $\mathfrak{B}(L', \#')$ is a reduction of $\mathfrak{B}(L, \#)$.*

In fact, the new chain equivalence $\bar{f}: \mathfrak{B}(L, \#) \to \mathfrak{B}(L', \#')$ may be defined as

$$\bar{f}[\alpha_1|\alpha_2|\ \dots\ |\alpha_p] = [f\alpha_1|f\alpha_2|\ \dots\ |f\alpha_p]. \tag{15}$$

3. The Abelian Complex.—For any abelian group Π, we construct the "abelian" complex of Π, by starting with the complex $A^0(\Pi) = K(\Pi, 1)$ with the product $*_0$ of (6) and applying the bar construction repeatedly to obtain new complexes

$$(A^1(\Pi), *_1) = \mathfrak{B}(A^0(\Pi), *_0),\ (A^t(\Pi), *_t) = \mathfrak{B}(A^{t-1}(\Pi), *_{t-1}). \tag{16}$$

Let $A(\Pi) = \mathfrak{B}^{\omega}(A^0(\Pi), *_0)$ be the union of all these complexes. This complex $A(\Pi)$ has in dimensions 1 and 2 just the cells $[x]$ and $[x, y]$ of $A^0(\Pi)$. In dimension 3, there are the cells $[x, y, z]$ of $A^0(\Pi)$, plus the new cell $[x|y]$ of $A^1(\Pi)$, with

$$\partial[x|y] = [x, y] - [y, x]. \qquad (17)$$

Thus the presence of this cell will make the generic cycle (4) a boundary. In dimension 4 there are the cells $[x, y, z, t]$, $[x, y|z]$, $[x|y, z]$, and $[x||y]$, with boundaries

$$\partial[x, y|z] = [\partial(x, y)|z] - [(x, y) *_0 z], \qquad (18)$$

$$\partial[x|y, z] = [x|\partial(y, z)] + [x *_0 (y, z)], \qquad (19)$$

$$\partial[x||y] = -[x *_1 y] = -[x|y] - [y|x]. \qquad (20)$$

In general, any cell σ of $A(\Pi)$ is a string of elements $x_1, \ldots, x_r$ of Π, separated by commas or by bars of various multiplicities. For the case when Π is the free abelian group F_a, the cell σ is *generic* provided that the elements $x_1, \ldots, x_r \in F_a$ which appear in σ are generic and pairwise disjoint. These generic cells again form a subcomplex $A(F_a^*)$.

THEOREM 3. *The generic abelian complex $A(F_a^*)$ has the integral homology groups*

$$H_1(A(F_a^*)) \cong F_a, \qquad H_q(A(F_a^*)) = 0, \qquad q > 1. \qquad (21)$$

The proof depends on first using the result of Theorem 1 to reduce $A^0(F_a^*)$ to a simpler complex G_0^0 in which the cells correspond exactly to the cycles (7). The homology groups of $\mathfrak{B}(G_0^0)$ may then be determined, much as in (7). This gives a reduction G_1^1 of $\mathfrak{B}(G_0^0)$; the result follows by iteration of this process.

4. The Cubical Complex.—In our previous study[3] of the effect of a single homotopy group π_m of a space upon the homology groups of that space, we had occasion to introduce for any abelian group Π a complex $Q(\Pi)$ in which the cells were cubical arrays of elements of Π, together with a normalization subcomplex $Q_N(\Pi)$, consisting of "slabs" and "diagonals" in these cubical arrays. For any abelian coefficient group G, the cubical cohomology groups of Π were defined as the relative cohomology groups of the pair $Q(\Pi)$, $Q_N(\Pi)$.

For the free group F_a we may again introduce the generic complex $Q(F_a^*)$ and its normalization subcomplex $Q_N(F_a^*) = Q_N(F_a) \cap Q(F_a^*)$.

THEOREM 4. *The integral homology groups for the generic cubical complex are*

$$H_1(Q(F_a^*), Q_N(F_a^*)) \cong F_a, \qquad H_q(Q(F_a^*), Q_N(F_a^*)) = 0, \qquad q > 1. \qquad (22)$$

In dimensions 1 and 2, $Q(\Pi)$ and $A(\Pi)$ are identical. This fact, plus the "generic acyclicity" expressed by (21) and (22), allows us to prove

THEOREM 5. *For any abelian group* Π, *the complex* $A(\Pi)$ *is chain equivalent to* $Q(\Pi)$ *modulo* $Q_N(\Pi)$.

Since this result still holds when $Q(\Pi)$ is replaced by any alternative construction enjoying the same formal properties (in particular, (22)), it follows that the homology and cohomology groups of $A(\Pi)$ depend not on the particular complex $A(\Pi)$, but essentially upon the fact that Π is an abelian group. We therefore call these groups the "abelian" homology and cohomology groups of Π; thus

$$A_q(\Pi) = H_q(A(\Pi)), \qquad A^q(\Pi;\ G) = H^q(A(\Pi);\ G) \tag{23}$$

for any coefficient group G. The groups of the complex $A^t(\Pi)$ will also be called the abelian homology and cohomology groups of Π on *level* t, in symbols

$$A_q^t(\Pi) = H_q(A^t(\Pi)), \qquad A^{q,t}(\Pi;\ G) = H^q(A^t(\Pi);\ G). \tag{24}$$

Among the cells of $A(\Pi)$ which are not in $A^t(\Pi)$ the cell $[x|_{t+1}y]$ has the lowest possible dimension $t + 3$. Hence the homology groups of $A^t(\Pi)$ agree with those of $A(\Pi)$ through dimension $t + 1$, so that

$$A_q^t(\Pi) \cong A^q(\Pi), \qquad A^{q,t}(\Pi;\ G) \cong A^q(\Pi;\ G), \qquad q \leq t + 1. \tag{25}$$

By Theorem 5, the abelian cohomology groups of Π are identical with the "cubical" cohomology groups of $Q(\Pi)$ modulo $Q_N(\Pi)$. We conjecture also that the levels of $A(\Pi)$ agree with the levels of $Q(\Pi)$, as introduced in our previous note,[3] so that

$$Q^{q,t}(\Pi;\ G) \cong A^{q,t}(\Pi;\ G). \tag{26}$$

We have established this result for $q = 1$, 2, and 3. In view of the geometric interpretation of the Q groups in Theorem 3 of our previous note, we have:

THEOREM 6. *If* X *is an arcwise connected topological space with a given homotopy group* $\pi_m (m > 1)$ *and with all other homotopy groups trivial, the singular cohomology groups of* X *in dimensions between* m *and* $2m - 1$, *and with any coefficient group* G, *are determined by* π_m *according to isomorphisms:*

$$H^{m-1+k}(X;\ G) = A^k(\pi_m;\ G), \qquad k = 1, \ldots, m.$$

If the conjecture (26) holds, we would have a similar isomorphism for all k, in the form $H^{m-1+k}(X;\ G) = A^{k,m-1}(\pi_m;\ G)$. In view of (25), this agrees with the result of the Theorem for low k.

5. *Explicit Homology Groups.*—The complex $A(\Pi)$ and its reductions may be used for explicit computations, with the aid of Theorem 2.

Let $\Pi = J$ be an infinite cyclic group. The ordinary cohomology groups

$H^q(J, G)$ are zero for $q > 1$; in fact, the usual proof of this fact may be used to show that $A^0(J)$ can be reduced to the complex U^0 which has just one cell, a cell $[1]$ of dimension 1, with boundary zero and product $[1] \underset{0}{*} [1] = 0$ of excess zero. An infinite sequence of bar constructions on U^0 yields, as in (16), a complex U^∞, and Theorem 2 shows that $A(J)$ is chain equivalent to U^∞. The complex U^∞ has only a finite number of cells in each dimension; hence any homology group of U^∞ may be computed in a finite number of steps. For example, if (n) denotes a cyclic group of order n and $+$ a direct sum, one has

$$A_1(J) = (\infty), A_2(J) = 0, A_3(J) = (2), A_4(J) = 0, A_5(J) = (2) + (3),$$
$$A_6(J) = 0, A_7(J) = (2) + (2), A_8(J) = 0, A_9(J) = (2) + (2) + (3) + (5),$$
$$A_{10}(J) = (2), A_{11}(J) = (2) + (2) + (2).$$

Let $\Pi = J/hJ$ be a cyclic group of order h. The usual computation[3] of the ordinary cohomology groups $H^q(\Pi, G) = H^q(A^0(\Pi), G)$ proves $A^0(\Pi)$ reducible to the complex $M^0(h)$ which has one cell $\{n\}$ in each dimension $n > 0$, with the boundary formulas

$$\partial\{2m\} = h\{2m - 1\}, \qquad \partial\{2m + 1\} = 0,$$

and which has a product of excess zero defined, using binomial coefficients, as

$$\{2m\} * \{2n\} = \binom{m+n}{n} \{2(m+n)\}, \qquad \{2m+1\} * \{2n+1\} = 0,$$

$$\{2m\} * \{2n+1\} = \{2m+1\} * \{2n\} = \binom{m+n}{n} \{2(m+n)+1\}.$$

Hence $A(J/hJ)$ is reducible to the complex $M^\infty(h)$ obtained from $M^0(h)$ by iterated bar construction. The latter complex again has but a finite number of cells in each dimension; explicit computation shows for instance that $A_3(J/hJ) = (3, h) + (2, h)$, where $(2, h)$ denotes a cyclic group of order the greatest common divisor of 2 and h.

If L and L' are complexes with products $\overset{1}{*}$, $\overset{1}{*}'$ of excess zero, one may form an "extended" *tensor product* complex $L \square L' = L \cup L' \cup (L \otimes L')$ in which the cells are cells σ of L, cells σ' of L', and new cells $\sigma \otimes \sigma'$ with dimension $d(\sigma) + d(\sigma')$. It is convenient to write $\sigma = \sigma \otimes \theta$, $\sigma' = \theta \otimes \sigma'$, where θ is the "void" cell with boundary 0. The boundary formulas are

$$\partial(\sigma \otimes \sigma') = (\partial\sigma) \otimes \sigma' + (-1)^{d(\sigma)} \sigma \otimes (\partial\sigma') \qquad (27)$$

and the product (with the conventions $\sigma \overset{1}{*} \theta = \sigma$, $\theta \overset{1}{*}' \sigma' = \sigma'$) is

$$(\sigma \otimes \sigma') \overset{1}{*} (\tau \otimes \tau') = (-1)^{d(\sigma')d(\tau)} (\sigma \overset{1}{*} \tau) \otimes (\sigma' \overset{1}{*}' \tau') \qquad (28)$$

THEOREM 7. *If K is reducible to L and K' to L', then $K \square L$ is reducible to $K' \square L'$.*

THEOREM 8. *For a direct sum $\Pi_1 + \Pi_2$ of abelian groups Π_1 and Π_2, $A^0(\Pi_1 + \Pi_2)$ is reducible to $A^0(\Pi_1) \square A^0(\Pi_2)$.*

Together with Theorem 2 and the results for cyclic groups, these Theorems prove

THEOREM 9. *If Π is a finitely generated abelian group, then any abelian homology or cohomology group $A_q(\Pi)$ or $A^q(\Pi, J)$ may be computed in a finite number of steps.*

In certain cases, the results can be put into invariant form. Thus for any abelian group Π (not necessarily finitely generated) we have

$$A_1(\Pi) \cong \Pi, \quad A_2(\Pi) = 0, \quad A_3(\Pi) \cong \Pi/2\Pi, \quad A_4(\Pi) \cong {}_2\Pi, \quad (29)$$

where ${}_2\Pi$ denotes the subgroup of all elements of order 2 in Π.

In this sense, the theory of generic acyclicity provides explicit methods for the computation of certain homology groups of the complex $K(\Pi, m)$ which gives the effect of the mth homotopy group of a space upon homology. If conjecture (26) holds, these results will apply to all homology groups of $K(\Pi, m)$.

* Essential portions of the study here summarized were done during the tenure of a John Simon Guggenheim Fellowship by one of the authors.

[1] Lyndon, Roger, *Ann. Math.*, **50**, 731–735 (1949).

[2] Eilenberg, S., and MacLane, S., PROC. NATL. ACAD. SCI., **36**, 443–447 (1950).

[3] Eilenberg, S., *Bull. Am. Math. Soc.*, **55**, 3–37 (1949), especially p. 22.

ARCS IN LOCALLY COMPACT GROUPS

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We outline the proof of a theorem useful in the topological investigation of groups: namely, that every locally compact group which is not totally disconnected contains an arc. With the aid of this theorem we are able to prove that every finite dimensional, locally compact group contains a one-parameter subgroup. These results accord with the conjecture that every locally compact group is a generalized Lie group.[1]

The results on finite dimensional groups were largely inspired by the work of Deane Montgomery.

1. The Topology of the Compact Subsets of a Locally Compact Space.—Let T be a locally compact space whose topology is defined by a fixed

uniform structure. We shall assume throughout that all uniformities are symmetrical. Let $\mathfrak{C}$ be the family of all compact subsets of T. For each uniformity α of T we can define a uniformity α' of $\mathfrak{C}$ by setting $(A, B) \in \alpha'$ if and only if (i) for each $a \in A$, there exists $b \in B$ such that $(a, b) \in \alpha$ and (ii) for each $b \in B$, there exists $a \in A$ such that $(a, b) \in \alpha$. It is easily verified that this family of uniformities determines a separated uniform structure for $\mathfrak{C}$; hence $\mathfrak{C}$ becomes a completely regular topological space. This definition is applicable to the family of all closed subsets of any uniform space, and in this form it becomes a generalization of the well-known topology of the closed subsets of a metric space. The space $\mathfrak{C}$ is compact or locally compact according as T is compact or locally compact, and it turns out that the topology of $\mathfrak{C}$ is independent of the choice of uniform structure for T. (This would be false if T were not locally compact.)

LEMMA 1. *Let $\mathfrak{A}$ be a family of compact subsets of T which is linearly ordered by inclusion and, as a subset of $\mathfrak{C}$, is closed and connected. Let $B = \cap_{A \in \mathfrak{A}} A$. If B is connected then every set $A \in \mathfrak{A}$ is connected.*

2. *Semigroups.*—By a semigroup G we shall mean an algebraic system with an associative binary operation and a unit element e. We write the operation by juxtaposition. In a semigroup it may happen that we can solve the equation $ax = e$ for some $a \neq e$. If this is the case for all a we have a group; however, we shall be interested in the opposite case. Suppose that from $a = axy$ follows $a = ax$. We shall say that such a semigroup is ordered because it is partially ordered by the relation $\geq$ where $a \geq b$ means that $a = bx$ for some $x \in G$. If S is a subsemigroup of an ordered semigroup G, then S is also ordered, but the ordering of S need not coincide with the ordering of G.

If G is also endowed with a Hausdorff topology such that the mapping $(a, b) \to ab$ of $G \times G$ into G is continuous, we say that G is a topological semigroup. Suppose that the topology of G is defined by a uniform structure. We shall say that the uniform structure is right invariant if, for every uniformity α, from $(a, b) \in \alpha$ follows $(ac, bc) \in \alpha$. A subset L of a topological semigroup is called a local subsemigroup if $e \in L$ and, for some neighborhood V of e in L, $VV \subset L$. The notions of local semigroup and local isomorphism are defined similarly following the usual definitions for groups.

LEMMA 2. *Let G be a locally compact, ordered semigroup. Assume that G has a right invariant uniform structure with a countable base. Let U be a compact neighborhood of the identity in G. Suppose that we can find sequences $x_1, x_2, \ldots$ of elements of G and $n_1, n_2, \ldots$ of integers such that $x_i \to e$ and $x_i^{n_i} \in U$. Then G contains a compact, connected, linearly ordered local subsemigroup L which contains more than one element.*

If A_i is the compact set $\{e, x_i, x_i^2, \ldots, x_i^{n_i - 1}\}$, then any limit (in the sense

of §1) of the sequence A_i satisfies the requirements for L. The requirement of countability is not actually necessary, but the proof becomes rather involved without it.

LEMMA 3. *Let L be a compact, connected, linearly ordered local semigroup with more than one element. Either L is locally isomorphic to the additive semigroup of non-negative real numbers or every neighborhood of e contains another idempotent.*

3. *Application to Groups.*—Let G be a locally compact group, and let $\mathfrak{C}$ be the family of its compact subsets. $\mathfrak{C}$ is a semigroup under multiplication in G, the unit element being the set $\{e\}$. Starting with the right invariant uniform structure of G, we define a uniform structure and topology for $\mathfrak{C}$ as in §1. It turns out that the uniform structure of $\mathfrak{C}$ is right invariant and that multiplication in $\mathfrak{C}$ is continuous. Let $\mathfrak{S}$ be the subsemigroup of those compact sets which contain e. $\mathfrak{S}$ is closed in $\mathfrak{C}$, so it is itself a locally compact semigroup. Furthermore, since $AB \supset A$ for $A, B \in \mathfrak{S}$, it is clear that $\mathfrak{S}$ is ordered.

Now suppose that G is metrizable and connected and contains more than one point. Let U be a compact neighborhood of e in G which does not contain all of G. Let $\mathfrak{U}$ be the family of compact subsets of U containing e. Then $\mathfrak{U}$ is a compact neighborhood of $\{e\}$ in $\mathfrak{S}$. Let X_i be a fundamental sequence of compact neighborhoods of the identity in G; then $X_i \to \{e\}$ in $\mathfrak{S}$. Since G is connected, for each i there is an integer n_i such that $X_i^{n_i} \in \mathfrak{U}$. All the conditions of Lemma 2 are satisfied by $\mathfrak{S}$; hence there exists in $\mathfrak{S}$ a compact, connected, linearly ordered local subsemigroup $\mathfrak{L}$ whose structure is given by Lemma 3. If idempotents appear in $\mathfrak{L}$, they are compact subgroups of G. All the elements of $\mathfrak{L}$ are connected subsets of G by Lemma 1. This proves

LEMMA 4. *Let G be a connected, locally compact, metrizable group containing more than one point. Either G contains arbitrarily small connected, compact subgroups larger than $\{e\}$ or there exists a family $F(t)$ $(0 \leq t \leq 1)$ of connected, compact subsets of G such that (i) $F(t) \neq e$ if $t > 0$, (ii) $\bigcap_{t>0} F(t) = F(0) = \{e\}$ and (iii) $F(t)F(u) = F(t+u)$ if $t + u \leq 1$.*

If G has finite dimension, then an infinite decreasing sequence of connected subgroups cannot exist,[2] so the second alternative must hold. Although we use it only to prove Theorem 1 below, the family $F(t)$ seems to be of considerable interest in itself. In a Lie group, the sets $F(t)$ can be chosen so that they are all neighborhoods of the identity $(t > 0)$. This is presumably possible for any locally connected group of finite dimension, but the author cannot prove it without assumptions of an analytic character.

THEOREM 1. *Every connected, locally compact group which contains more than one element contains an arc.*

If G has a family of sets $F(t)$ as described in Lemma 4, we can construct

an arc in the same way that one constructs an isometric arc in a convex metric space. If G contains a compact, connected subgroup, then it contains even a one-parameter subgroup. There remains only the non-metrizable case. Here G has a compact normal subgroup N such that G/N is metrizable. Either N contains an arc or N is totally disconnected and we can "raise" an arc of G/N.

The set of elements which can be joined to the identity by an arc in G is a normal subgroup $G\dagger$. It is a reasonable conjecture that $G\dagger$ is dense. One could prove this by showing that an arc of $G/G\dagger^-$ could be "raised" into G.

4. Finite Dimensional Groups.—LEMMA 5. *Let X be a compact set of dimension r in a locally arcwise-connected metric group. Then either X has an interior point or there exists an arc A such that dim $(AX) > r$.*

This lemma is essentially due to Montgomery.[2] Our proof, which uses homotopy theory, follows the intuition closely.

THEOREM 2. *Let G be a locally arcwise-connected group of dimension n. Then any compact subset of G whose dimension is n has an interior point.*

THEOREM 3. *Let G be a locally arcwise-connected group of finite dimension. Then G is locally compact.*

In particular, a locally connected, complete, metric group of finite dimension is locally compact.

We return to the consideration of $G\dagger$, the arcwise-connected subgroup of G. We can define a new and stronger topology for G under which G becomes locally arcwise-connected and $G\dagger$ becomes the connected component of the identity: If V is a neighborhood of the identity in G, then the arcwise-connected component of the identity in V is taken as a new neighborhood. We shall denote by G^* the group $G\dagger$ taken with this new topology. If G is metrizable, so is G^*. If G has finite dimension, then so does G^* because it has a faithful representation in G. In this case G^* is locally compact by Theorem 3; in general, however, G^* need not be locally compact.

These considerations apply to any arcwise connected subgroup of a finite dimensional group and give

THEOREM 4. *Any arcwise connected subgroup of a Lie group is analytic.*

LEMMA 6. *A locally connected group of finite dimension greater than one contains a proper connected closed subgroup.*

This lemma has been proved by Montgomery for locally Euclidean groups.[3] Our argument follows his using Theorem 2 in place of the Brouwer theorem of invariance of domain.

THEOREM 5. *Every connected, locally compact group of finite positive dimension contains a one-parameter subgroup.*

The proof is by induction on the dimension n of G. If $n = 1$, then G^* is locally arcwise-connected and of dimension 1. By Theorem 2 it

must be locally Euclidean and itself a one-parameter group. Then G^* determines a one-parameter subgroup of G. Suppose that $k > 1$ and that the theorem is true for $n < k$. If G has dimension k and is locally connected, then we can find a one-parameter subgroup of the subgroup guaranteed by Lemma 6, whose dimension is positive and less than k. Finally suppose that G has dimension k but is not locally connected. Then G^* is a locally compact, locally connected group of positive dimension at most k, so G^* contains a one-parameter subgroup, which in turn gives us a one-parameter subgroup of G.

[1] Gleason, A., "On the Structure of Locally Compact Groups," PROC., NATL. ACAD. SCI., **35**, 384–386 (1949). Iwasawa, K., "On Some Types of Topological Groups," *Ann. Math.*, **50**, 507–558 (1949).

[2] Montgomery, D., "Theorems on the Topological Structure of Locally Compact Groups," *Ann. Math.*, **50**, 570–580 (1949).

[3] Montgomery, D., "A Theorem on Locally Euclidean Groups," *Ibid.*, **48**, 650–658 (1947).

PSEUDO-CONFORMAL GEOMETRY OF POLYGENIC FUNCTIONS OF SEVERAL COMPLEX VARIABLES

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1. A complex function

$$w = F(z^\alpha) = F(z^1, \ldots, z^n) = \phi(x^\alpha; y^\alpha) + i\psi(x^\alpha; y^\alpha), \qquad (1)$$

where the real ϕ and ψ are single-valued continuous functions and possess continuous partial derivatives over a region R of $2n$ dimensional real space Σ_{2n} of coordinates $(x^1, \ldots, x^n;\ y^1, \ldots, y^n)$ is termed a *polygenic function*[1] of the n complex variables $z^\alpha = x^\alpha + iy^\alpha$, where $\alpha = 1, \ldots, n$.

We shall study the first derivatives of a polygenic function and also present some new results in pseudo-conformal geometry.

2. For the class of polygenic functions, the linear operators

$$\frac{\partial}{\partial z^\alpha} = \frac{1}{2}\left(\frac{\partial}{\partial x^\alpha} - i\frac{\partial}{\partial y^\alpha}\right); \quad \frac{\partial}{\partial \bar{z}^\alpha} = \frac{1}{2}\left(\frac{\partial}{\partial x^\alpha} + i\frac{\partial}{\partial y^\alpha}\right), \qquad (2)$$

are important. These are called the *mean* and *phase derivatives*, respectively. The operations $\dfrac{\partial}{\partial z^\alpha}$ and $\dfrac{\partial}{\partial \bar{z}^\alpha}$ are not partial derivatives but signify the application of the linear operators (2).

3. A polygenic function w is monogenic over R if and only if

$$\frac{\partial w}{\partial \bar{z}^\alpha} = 0, \qquad \text{where } \alpha = 1, \ldots, n. \tag{3}$$

These are equivalent to the $2n$ Cauchy-Riemann equations. The real and imaginary parts obey the n^2 Poincaré partial differential equations of second order

$$\frac{\partial^2 w}{\partial z^\alpha \partial \bar{z}^\beta} = 0, \qquad \text{where } \alpha, \beta = 1, \ldots, n. \tag{4}$$

A *multiharmonic function* is any solution w of this Poincaré system.[2] If $w = \phi + i\psi$ is monogenic over R, then ϕ and ψ are *conjugate-multiharmonic* over R.

4. The locus of points in Σ_{2n}, defined by the equations

$$z^\alpha = f^\alpha(Z^\beta), \tag{5}$$

where the n f^α are monogenic functions over a region of the $2m$ dimensional parametric space Σ'_{2m} of the m complex variables $Z^\beta = X^\beta + iV^\beta$, where $\beta = 1, \ldots, m$, such that the jacobian-matrix

$$\left(\frac{\partial x^\alpha}{\partial X^\beta}, \quad \frac{\partial x^\alpha}{\partial V^\gamma}; \quad \frac{\partial y^\alpha}{\partial X^\beta}, \quad \frac{\partial y^\alpha}{\partial V^\gamma}\right), \tag{6}$$

is of rank $2m$, is called a *pseudo-conformal manifold* Σ_{2m} of $2m$ dimensions. Evidently $m \leqq n$.

If $m = n$, there results a correspondence, called a *pseudo-conformal transformation* T, between the two pseudo-conformal manifolds Σ_{2n} and Σ'_{2n}. All such maps T form the *pseudo-conformal group* G. The associated geometry is termed *pseudo-conformal geometry*.

Under G, any Σ_{2m} becomes a Σ_{2m}. For $m = 1$, any Σ_2 is called a *conformal or analytic surface*. The reason for this terminology is that G induces conformality between pairs of analytic surfaces.

The angle λ_α between any direction at a fixed point P on a conformal surface S and its orthogonal projection on the z^α-plane depends only on the position of the point P on S. Moreover the sum of the squares of the cosines of the resulting n angles $\lambda_1, \ldots, \lambda_n$, at P, is unity.[3]

An area A on a conformal surface S is the sum of the projected areas A_α on the z^α-planes.

5. Consider a polygenic function w defined over a conformal surface S in R. If $Z = X + iV$ is the parameter describing S, then

$$\frac{dw}{dZ} = \left(\frac{\partial w}{\partial z^\alpha}\frac{dz^\alpha}{dZ}\right) + \left(\frac{\partial w}{\partial \bar{z}^\alpha}\frac{d\bar{z}^\alpha}{d\bar{Z}}\right)e^{-2i\theta}, \tag{7}$$

in which the repeated index α means to sum with respect to that index from 1 to n. It is noted that θ is the angle between a direction at a point P on S and the parametric curve Y = const., through P on S. Representing dw/dZ in a plane, called the *derivative plane* Δ, dw/dZ is depicted as a clock[4] with center vector $H + iK = \dfrac{\partial w}{\partial z^\alpha}\dfrac{dz^\alpha}{dZ}$, and phase vector $h + ik = \dfrac{\partial w}{\partial \bar{z}^\alpha}\dfrac{d\bar{z}^\alpha}{\partial Z}$. This clock depends on the point P, the conformal surface S through P, and the parameter Z describing S.

If a change of the parameter Z is performed, the central and phase vectors of the clock Γ are multiplied by the same real number $\rho > 0$ and rotated through equal angles but in opposite directions. Denote this operation on a clock Γ by $S^*(\Gamma)$. Then all the clocks corresponding to a fixed point P and a fixed surface S through P are the ∞^2 clocks $S^*(\Gamma)$.

The totality of clocks at a given point P is ∞^{2n} but essentially there are ∞^{2n-2}, one to each conformal surface S through P.

If $\Gamma_1, \ldots, \Gamma_n$ are n clocks belonging to n distinct conformal surfaces $S_1, \ldots, S_n$, not all contained in the same Σ_{2n-2}, then any clock Γ at P is given by

$$\Gamma = S_1^*(\Gamma_1) + \ldots + S_n^*(\Gamma_n). \tag{8}$$

Thus the totality of clocks at a given point P forms an abstract vector space of n dimensions.

A set of m clocks $\Gamma_1, \ldots, \Gamma_m$, at a given point P, is found to be linearly dependent if and only if they are obtained from m conformal surfaces $S_1, \ldots, S_m$, through P, all of which belong to the same pseudo-conformal manifold Σ_{2m-2}.

A polygenic function w is monogenic at a given point if and only if dw/dZ is unique along each of n distinct conformal surfaces through P, not all contained in the same Σ_{2n-2}.

This result generalizes the usual definition of a monogenic function.

A polygenic function w is multiharmonic in Σ_{2n} if and only if it is multiharmonic over every Σ_{2m} where m is fixed and $m \leqq n$.

Also it may be shown that the center transformation is direct conformal for every S if and only if w is multiharmonic in Σ_{2n}.

6. The *pseudo-angle* between a $(2n - 1)$ dimensional manifold S_{2n-1}: $F(x^1, \ldots, x^n; y^1, \ldots, y^n) = 0$, and a curve $Cx^\alpha: = x^\alpha(t)$, where $\alpha = 1, \ldots, n$, at a given point P of intersection, is

$$\theta = \arctan \frac{\dfrac{\partial F}{\partial x^\alpha}dx^\alpha + \dfrac{\partial F}{\partial y^\alpha}dy^\alpha}{-\dfrac{\partial F}{\partial y^\alpha}dx^\alpha + \dfrac{\partial F}{\partial x^\alpha}dy^\alpha}. \tag{9}$$

The pseudo-angle characterizes the pseudo-conformal group G.[5]

Let S_{2m} be a fixed $2m$ dimensional manifold contained in Σ_{2n} so that $m \leqq n$. Let P be an arbitrary point of S_{2m}. Suppose that S_{2n-1} is an arbitrary $(2n-1)$ dimensional manifold in Σ_{2n} which contains P, and let the intersection of S_{2m} and S_{2n-1} be an S_{2m-1}. Thus S_{2m} is not contained in S_{2n-1}.

The pseudo-angle between any curve C, in S_{2m}, and S_{2n-1} at P, is equal to that between C and S_{2m-1}, for every S_{2n-1}, if and only if S_{2m} is pseudo-conformal.

This gives a geometric characterization of the pseudo-conformal manifolds Σ_{2m} contained in a given pseudo-conformal manifold Σ_{2n}, where $m \leqq n$.

If $w = \phi + i\psi$ is monogenic over R, then the curves of $\phi = const.$ are pseudo-orthogonal to the manifolds $\psi = const.$

For $n = 1$, this reduces to the well-known result that the components of a monogenic function of a complex variable give rise to an orthogonal isothermal net.

[1] The term polygenic was introduced by Kasner in 1927. See: "A New Theory of Polygenic (or non-Monogenic) functions," *Science*, **66**, 581–582 (1927). The term non-analytic is also used.

[2] Poincaré, *Compt. rend.*, **96**, 238, (1883); *Acta Math.*, **2**, 99, (1883), **22**, 112 (1898); *Palermo Rendiconti* (1907).

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[5] De Cicco, "The Pseudo-Angle in Space of $2n$ Dimensions," *Bull. Am. Math. Soc.*, **51**, 102–174 (1945). Also "Functions of Several Complex Variables and Multiharmonic Functions," *Am. Math. Monthly*, **56**, 315–325 (1949).

THE ELEMENT OF VOLUME OF THE ROTATION GROUP

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The n-dimensional rotation group is an $\frac{n(n-1)}{2}$-parameter group and if we set $n = 2k$ or $n = 2k + 1$, according as n is even or odd, it is usually convenient to adopt as k of these parameters the k angles $\theta_1, \theta_2, \ldots, \theta_k$ which determine the class of the group to which the particular element X of the group which we wish to specify belongs (the function of the remain-

ing $\frac{n(n-1)}{2} - k$ parameters being, then, to locate the element X in its class). The element of volume of the rotation group appears, when this is done, as a product of two factors in which one of the factors involves only the parameters $\theta_1, \ldots, \theta_k$ (which serve to specify the class) and their differentials and the other factor involves the remaining $\frac{n(n-1)}{2} - k$ parameters and their differentials. We shall refer to the first of these factors as the class factor (the other being the non-class factor). Whenever the function which we wish to integrate over the group is a class function it is sufficient to know the class factor of the element of volume of the group (the other factor canceling out in the process of averaging over the group) and, so far as we know, it is only the class factor of the element of volume of the n-dimensional rotation group that has been furnished to date. We propose to give in the present note the complete expression of the element of volume of the n-dimensional rotation group. The calculations necessary to furnish this yield at the same time the element of volume of the n-dimensional Lorentz group and of the n-dimensional quasi-Lorentz groups. The parameters we shall use are the analogs for the n-dimensional rotation group of the familiar Eulerian angles ϕ, θ, ψ (for the 3-dimensional rotation group); these have certain advantages (in the physical applications) over the class and non-class parameters (which are, from the theoretical point of view, more attractive) and the element of volume of the 3-dimensional rotation group in terms of the Eulerian angles, namely, $\sin\theta\, d\phi\, d\theta\, d\psi$ is already well known and widely used. The progress made in the present note is the extension of this result to the general value of n; the case $n = 4$ is of particular interest in view of the intimate connection between the 4-dimensional rotation group and the 4-dimensional Lorentz group.

Any n-dimensional rotation matrix X may be written as the product of $\frac{n(n-1)}{2}$ plane rotation matrices. We indicate by subscripts the plane in which the rotation takes place; thus $R_{12}(\phi)$ denotes the rotation $n \times n$ matrix of which the elements in the first two rows and first two columns are $\begin{pmatrix} \cos\phi & -\sin\phi \\ \sin\phi & \cos\phi \end{pmatrix}$, the remaining elements being zero, save the diagonal elements which are 1. Of the $\frac{n(n-1)}{2}$ angles involved, one in each of the plane rotation matrices whose product is X, $n-1$ are longitude (or equatorial) angles $\phi_1, \phi_2, \ldots, \phi_{n-1}$, each of which lies in the interval $-\pi \leq \phi < \pi$ while the remainder are latitude (or meridian) angles $\theta_1, \theta_2, \ldots, \theta_{(n-1)(n-2)/2}$ each of which lies in the interval $0 \leq \theta \leq \pi$. In the cases $n = 3$, 4 and 5, for example, we have

$$X = R_{12}(\phi_2)R_{23}(\theta_1)R_{12}(\phi_1); \quad n = 3;$$
$$X = R_{12}(\phi_3)R_{23}(\theta_3)R_{34}(\theta_2)R_{12}(\phi_2)R_{23}(\theta_1)R_{12}(\phi_1); \quad n = 4;$$
$$X = R_{12}(\phi_4)R_{23}(\theta_5)R_{34}(\theta_4)R_{45}(\theta_3)R_{12}(\phi_3)R_{23}(\theta_3)R_{34}(\theta_2)R_{12}(\phi_2)R_{23}(\theta_1)R_{12}(\phi_1); \quad n = 5.$$

It helps in the calculations to observe that, except for the change in the dimensions of the plane rotation matrices involved, each of these expressions involves the preceding expression as a factor. Thus if we denote, for a moment, the typical element of the n-dimensional rotation group by X_n, so that $X_2 = R_{12}(\phi_1)$, X_3 is the product of X_2 (increased to dimension 3) by $R_{12}(\phi_2)R_{23}(\theta_1)$. Similarly X_4 is the product of X_3 (increased to dimension 4) by $R_{12}(\phi_3)R_{23}(\theta_3)\ R_{34}(\theta_2)$, X_5 is the product of X_4 (increased to dimension 5) by $R_{12}(\phi_4)R_{23}(\theta_5)R_{34}(\theta_4)R_{45}(\theta_3)$ and so on. The net result of this is that the element of volume of the n-dimensional rotation group involves the element of volume of the $(n-1)$-dimensional rotation group as a factor. Thus the element of volume of the 2-dimensional rotation group is $d\phi_1$; the element of volume of the 3-dimensional rotation group is the product of this by $\sin\theta_1\, d\theta_1\, d\phi_2$, namely, $\sin\theta_1\, d\phi_1\, d\theta_1\, d\phi_2$. The element of volume of the 4-dimensional rotation group is the product of the element of volume of the 3-dimensional rotation group by $\sin^2\theta_2 \sin\theta_3\, d\theta_2\, d\theta_3\, d\phi_3$, namely, $\sin\theta_1 \sin^2\theta_2 \sin\theta_3\, d\phi_1\, d\theta_1\, d\phi_2\, d\theta_2\, d\theta_3\, d\phi_3$. Similarly, the element of volume of the 5-dimensional rotation group is the product of the element of volume of the 4-dimensional rotation group by $\sin^3\theta_4 \sin^2\theta_5 \sin\theta_6\, d\theta_4\, d\theta_5\, d\theta_6\, d\phi_4$ and so on. The final result may, therefore, be stated as follows:[1]

The element of volume of the n-dimensional rotation group is obtained by multiplying the product of the differentials of the $\dfrac{n(n-1)}{2}$ angles ϕ and θ by the product of $n-2$ factors of which the first is $\sin\theta_1$, the second $\sin^2\theta_2 \sin\theta_3$, the third $\sin^3\theta_4 \sin^2\theta_5 \sin\theta_6$, and so on, the last being $\sin^{n-2}\theta_p \sin^{n-3}\theta_{p+1} \cdots \sin\theta_{p+n-3}$ where $p = \dfrac{(n-2)(n-3)}{2} + 1$ so that $p + n - 3 = \dfrac{(n-1)(n-2)}{2}$.

For the case of the Lorentz group $\sin\theta_{n-2}$ must be replaced by $\sinh\theta_{n-2}$, the range of θ being over all non-negative values. Thus the element of volume of the 4-dimensional Lorentz group is $\sin\theta_1 \sinh^2\theta_2 \sin\theta_3\, d\phi_1\, d\theta_1\, d\phi_2\, d\theta_2\, d\theta_3\, d\phi_3$, the parametric space being furnished by the formulas $-\pi \leq \phi_1 < \pi$; $0 \leq \theta_1 \leq \pi$; $-\pi \leq \phi_2 < \pi$; $0 \leq \theta_2$; $0 \leq \theta_3 \leq \pi$; $-\pi \leq \phi_3 < \pi$. Similarly, for the 4-dimensional quasi-Lorentz group, the element of volume is $\sin\theta_1 \sinh^2\theta_2 \sinh\theta_3\, d\phi_1\, d\theta_1\, d\phi_2\, d\theta_2\, d\theta_3\, d\phi_3$ where, now, both θ_2 and θ_3 range over all positive values.

[1] Details of the calculations will appear in *"Lectures on Matrices and Matrix Groups"* to be published by the Centro de Pesquisas Fisicas, Rio de Janeiro, Brazil.

SCHWARZ' INEQUALITY AND LORENTZ SPACES

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The most fundamental formula in the theory of Euclidean or unitary metric spaces is, doubtless, Schwarz' inequality which states that the modulus of the scalar product of two vectors, real or complex, is dominated by the product of their magnitudes (the modulus of the scalar product being equal to the product of the magnitudes when, and only when, the two vectors are linearly dependent). Expressed in matrix notation $(x^*y)(y^*x) \leqslant (x^*x)(y^*y)$ where x and y are any two $n \times 1$ matrices, real or complex, and x^*, y^* are the conjugate transposed matrices of x and y, respectively. The proof, now classical, of this fundamental result runs as follows. If λ is any complex number the squared magnitude of $\lambda x + y$ is never negative; on varying the argument of λ it follows that neither of the two quadratic polynomial functions of $|\lambda|$: $(x^*x)|\lambda|^2 \pm 2|x^*y||\lambda| + (y^*y)$ is ever negative. Hence the quadratic polynomial function $(x^*x)\epsilon^2 + 2|x^*y|\epsilon + (y^*y)$ of the real variable ϵ is never negative and this implies, on using the theory of the zeros of a quadratic polynomial, Schwarz' inequality. We wish to point out in the present note that the positiveness of x^*x and the theory of the zeros of a quadratic polynomial are really somewhat foreign to Schwarz' fundamental inequality and to give the analog of Schwarz' inequality for Lorentz spaces, in which the scalar product of a non-zero $n \times 1$ matrix by itself may be negative or zero, instead of being always positive as in Euclidean or unitary spaces.

Let x_1 and x_2 be any two linearly independent $n \times 1$ matrices, real or complex, and denote by X the $n \times 2$ matrix whose column matrices are x_1 and x_2. If x_1 and x_2 are linear combinations of two other $n \times 1$ matrices, y_1 and y_2 (necessarily linearly independent), we have $X = YA$ where Y is the $n \times 2$ matrix whose column matrices are y_1 and y_2 and A is a non-singular 2×2 matrix. We take y_1 to be a multiple of x_1, so chosen that $y_1^*y_1 = 1$ and we set $y_2 = -(y_1^*x_2)y_1 + x_2$. Then $y_1^*y_2 = 0$ and y_2 is not the zero matrix. The 2×2 matrix Y^*Y, being the matrix $\begin{pmatrix} 1 & 0 \\ 0 & y_2^*y_2 \end{pmatrix}$, has a positive determinant and, since $X^*X = A^*(Y^*Y)A$, the determinant of the 2×2 matrix X^*X, being the product of the determinant of the 2×2 matrix Y^*Y by $|\det A|^2$, is positive. Since X^*X is the matrix $\begin{pmatrix} x_1^*x_1 & x_1^*x_2 \\ x_2^*x_1 & x_2^*x_2 \end{pmatrix}$ this proves Schwarz' inequality in the case where the two $n \times 1$ matrices x_1 and x_2 are linearly independent. When they are linearly dependent X may be written in the form YA where the 2×2

matrix A is singular. Hence the 2×2 matrix X^*X is singular; this completes the proof of Schwarz' inequality.

Before passing to the case of Lorentz spaces we make the obvious remark that if we wish to avail ourselves of the diagonalization property of Hermitian matrices the above proof of Schwarz' inequality may be given in the following (less elementary and only slightly more concise) variant form. The 2×2 matrix X^*X is Hermitian and so there exists a 2×2 unitary matrix U such that $U^*(X^*X)U$ is diagonal. Since X^*X is positively definite the diagonal elements of this diagonal form of X^*X (i.e., the characteristic numbers of X^*X) are positive and hence the determinant of X^*X is positive. This proves the part of Schwarz' inequality which is concerned with linearly independent $n \times 1$ matrices x_1 and x_2. The simplest and most direct proof of the part which is concerned with linearly dependent $n \times 1$ matrices x_1 and x_2 is that given above.

Turning now to the case of Lorentz spaces, let F be the diagonal $n \times n$ matrix all of whose diagonal elements are 1 save the last which is -1. The Lorentz scalar product of two $n \times 1$ matrices x and y is y^*Fx and we say that the $n \times 1$ matrix x, real or complex, is time-like, null or space-like according as x^*Fx is negative, zero or positive, respectively. The last element of a time-like $n \times 1$ matrix cannot be zero and it follows that every pair of linearly independent $n \times 1$ matrices x_1 and x_2 contains a space-like linear combination; this implies that if $x_1^*Fx_1 \leqslant 0$ and $x_1^*Fx_2 = 0$ then $x_2^*Fx_2 > 0$. Let us denote by X the $n \times 2$ matrix whose column matrices are any given pair of linearly independent matrices x_1 and x_2; if x_1 and x_2 are linear combinations of two other $n \times 1$ matrices y_1 and y_2, necessarily linearly independent, we have $X = YA$ where Y is the $n \times 2$ matrix whose column matrices are y_1 and y_2 and A is a non-singular 2×2 matrix. Hence the determinant of the 2×2 matrix X^*FX has the same sign as the determinant of the matrix Y^*FY and, if x_1 and x_2 are linearly dependent, the matrix X^*FX is singular. Taking x_1 and x_2 to be linearly independent there exists a linear combination y_1 of x_1 and x_2 which is such that $y_1^*Fy_1 = 1$ (since there exists a space-like linear combination of x_1 and x_2). We may suppose that y_1 is not a multiple of x_2 (since it is not a multiple of both x_1 and x_2) and we set $y_2 = -(y_1^*Fx_2)y_1 + x_2$. Then $y_1^*Fy_2 = 0$ and the determinant of the 2×2 matrix Y^*FY is $y_2^*Fy_2$. Thus, if there exists no time-like linear combination of x_1 and x_2, the determinant of the 2×2 matrix X^*FX is non-negative. Similarly, if there does exist a time-like linear combination of x_1 and x_2, this determinant is negative. Thus we have the following analog, for Lorentz spaces, of Schwarz' inequality:

$$(x_1^*Fx_1)(x_2^*Fx_2) < |x_1^*Fx_2|^2$$

if, and only if, there exists a time-like linear combination of the two $n \times 1$ matrices x_1 and x_2, supposed linearly independent.

Since there always exists a space-like linear combination of two linearly independent $n \times 1$ matrices, x_1 and x_2, one of the two characteristic numbers of the 2×2 Hermitian matrix X^*FX is positive. The second characteristic number may be either positive, zero or negative; in the first case, i.e., when the 2×2 Hermitian matrix X^*FX is positively definite, we say that the $n \times 2$ matrix X is space-like (so that every non-trivial linear combination of the column matrices of a space-like $n \times 2$ matrix is space-like). Thus we may complete the analog, for Lorentz spaces, of Schwarz' inequality as follows:

If $(x_1^*Fx_1)(x_2^*Fx_2) = |x_1^*Fx_2|^2$ there exists a null non-trivial linear combination of x_1 and x_2 while there does not exist a time-like linear combination of x_1 and x_2; $(x_1^*Fx_1)(x_2^*Fx_2) > |x_1^*Fx_2|^2$ if, and only if, the $n \times 2$ matrix X, whose column matrices x_1 and x_2 are linearly independent, is space-like.

These results may be extended to the case of p $n \times 1$ matrices $x_1, \ldots, x_p$ where $3 \leqslant p \leqslant n$. The proof given above shows that the $p \times p$ matrix X^*FX, where X is the $n \times p$ matrix whose column matrices are $x_1, \ldots, x_p$, is singular if the p $n \times 1$ matrices $x_1, \ldots, x_p$ are linearly dependent and so we shall suppose that they are linearly independent. The $p \times p$ matrix X^*FX cannot have less than $p - 1$ positive characteristic numbers (since every pair of linearly independent $n \times 1$ matrices possesses a space-like linear combination). Thus the $n \times p$ matrix X is space-like (by which we mean that every non-trivial linear combination of its column matrices is space-like) if, and only if, the determinant of the $p \times p$ matrix X^*FX is positive. This determinant is negative if, and only if, there exists a time-like linear combination of the p $n \times 1$ matrices $x_1, \ldots, x_p$. The matrix X^*FX is singular if, and only if, there exists a null non-trivial linear combination of $x_1, \ldots, x_p$ (while no time-like linear combination of these exists). In the case $p = n$ the determinant of X^*FX is negative since there certainly exists a time-like linear combination of n linearly independent $n \times 1$ matrices $x_1, \ldots, x_n$; the negativeness of $\det X^*FX$ is *a priori* evident since $\det X^*FX$ is the product of $\det F$ by $|\det X|^2$.

If we shall ever have to consider physical spaces with more than one time direction we must introduce quasi-Lorentz spaces. Thus, for example, if we have two time-directions the $n \times n$ diagonal matrix G whose first $n - 2$ diagonal elements are 1, the last two being -1, takes the place of F for Lorentz spaces and of the $n \times n$ identity matrix E for Euclidean, or unitary, spaces. If we have q time directions G is the $n \times n$ diagonal matrix whose first $n - q$ diagonal elements are 1, the remaining q being -1; there is no lack of generality involved in taking $q \leqslant n/2$. An $n \times 1$ matrix x, real or complex, is space-like, null or time-like according as

x^*Gx is positive, zero or negative, respectively. If $x_1, \ldots, x_p$ are p linearly independent $n \times 1$ matrices the $n \times p$ matrix X is said to be space-like, null or time-like according as every non-trivial linear combination of $x_1, \ldots, x_p$ is space-like, null or time-like, respectively; thus X is time-like if, and only if, the $p \times p$ matrix X^*GX is positively definite; X is null if, and only if, X^*GX is the zero $p \times p$ matrix and X is time-like if, and only if, X^*GX is negatively definite. Every set of $q + 1$ linearly independent $n \times 1$ matrices contains a space-like linear combination and every set of $n - q + 1$ linearly independent $n \times 1$ matrices contains a time-like linear combination. If, then, $p > q$ the $p \times p$ matrix X^*GX cannot have less than $p - q$ positive characteristic numbers and, similarly, if $p > n - q$, X^*GX cannot have less than $p + q - n$ negative characteristic numbers. Thus, if $p = n$, X^*GX has exactly $n - q$ positive characteristic numbers and exactly q negative characteristic numbers. If $p = n - 1$ the sign of one of the characteristic numbers of X^*GX is uncertain (there being at least $n - q - 1$ positive characteristic numbers and at least $q - 1$ negative characteristic numbers). It follows that the necessary and sufficient condition that the space spanned by $n - 1$ linearly independent matrices $x_1, \ldots, x_{n-1}$ should have a q-dimensional time-like subspace is that the determinant of the $(n - 1) \times (n - 1)$ matrix X^*GX should have the sign of $(-1)^q$ and the necessary and sufficient condition that this space should have a $(n - q)$-dimensional space-like subspace is that this determinant should have the sign of $(-1)^{q-1}$. If $p = n - 2$, and $q \geqslant 2$, the signs of two of the characteristic numbers of X^*GX are uncertain and the necessary and sufficient condition that the space spanned by the $n - 2$ linearly independent $n \times 1$ matrices $x_1, \ldots, x_{n-2}$ should have a $(q - 1)$-dimensional time-like subspace and a $(n - q - 1)$-dimensional space-like subspace is that the determinant of X^*GX should have the sign of $(-1)^{q-1}$. If $q < p \leqslant n - q$ the matrix X^*GX has $p - q$ positive characteristic numbers and the signs of the remaining q characteristic numbers are uncertain. Thus, if $q = 2$, the necessary and sufficient condition that the space spanned by the p linearly independent $n \times 1$ matrices $x_1, \ldots, x_p$ should possess a $(p - 1)$-dimensional space-like subspace and that there should exist a time-like linear combination of $x_1, \ldots, x_p$ is the negativeness of the determinant of the $p \times p$ matrix X^*GX, $2 < p \leqslant n - 2$.

THE CHEMICAL NATURE OF THE ACROSOME IN THE MALE GERM CELLS

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The close connection of the acrosome of the animal sperm with the Golgi apparatus was first suggested by Bowen[1] in 1923, who stated that the acrosomal material might be "secreted" from the Golgi apparatus. While his cytological studies seem to support the concept that the acrosome is derived from the Golgi apparatus, the staining methods employed do not allow any conclusions as to the chemical nature of either structure. The recent development of cytochemical procedures, using specific staining reactions for the chemical characterization of cellular constituents *in situ* now makes possible an analysis of some of the chemical components of the dictyosomal material[22] and the acrosome of the male germ cells. *Arvelius albopunctatus*, an hemipteran insect, provides an especially favorable material for the cytochemical studies, since the different lobes of the testis are characterized by a constant and marked difference in the size of their spermatocytes, spermatids and sperms.[2, 19, 20] In the present paper evidence is presented that the acrosome of the sperm is derived from the dictyosomes of the primary spermatocytes, and that both contain polysaccharides in a 1,2 glycol grouping. The amount of polysaccharides in the dictyosomal material and in the acrosome of spermatocytes, spermatids and sperms is strikingly higher in the large sized cells of the third and fifth lobes than in the normal and small sized cells of the remaining four lobes. For the identification of the polysaccharides in the cells of the testis we used the microchemical periodic acid Schiff (PAS) reaction resulting in the staining of polysaccharide structures in tissues as described by Hotchkiss[5] in 1948. According to Hotchkiss[5] and McManus,[12] the reaction of periodic acid with carbohydrates, when present as 1,2 glycol grouping, is considered to be the following:

$$\mathrm{R{-}\overset{OH}{\overset{|}{C}}{-}\overset{OH}{\overset{|}{C}}{-}R + HIO_4 \rightarrow 2R{-}CHO}$$

The aldehydes which are formed after periodic acid oxidation from 1,2 glycols in sections form a colored complex with the Schiff reagent. In order to characterize the type of polysaccharides in our tissues, we used the Hotchkiss reaction in combination with the acetylation technique of McManus and Cason[13] and with enzymes such as amylase, diastase and various hyaluronidases (derived from bull testis and from bacteria).[23]

Furthermore additional specific staining for desoxyribosenucleic acid (DNA) by means of methyl-green,[14] and for basic proteins by means of fast-green[20] was secured simultaneously in the same sections. The detailed technique of these procedures as well as a standardization of the Hotchkiss reaction for quantitative estimation of polysaccharides in tissues will be described in another publication (in collaboration with Orbison and Lieb).

The testes of Arvelius[24] were fixed in Carnoy's acetic-alcohol and sectioned at 6, 10 and 16 microns. Staining was always performed in the same way under standardized conditions, and enzyme experiments, such as for instance the treatment with hyaluronidase, were made with sections directly adjacent to the control section (without periodic acid) and to the test slide (with periodic acid but without the enzyme). The amount of polysaccharides was judged on the basis of the intensity of the Schiff color after periodic acid oxidation in individual cells by the photometric microscopic method, as described by Schrader and Leuchtenberger[20] and with an apparatus of the type designed by Pollister and Moses.[15] Control sections, without periodic acid oxidation, did not show any development of color after exposure to the Schiff reagent. For the absorption measurements of the Schiff color in the sections, acrosomal material of spherical shape was selected in large and in normal sized cells which were in the same stage of development and were present in the same slide. Photometric measurements of the acrosome in the small sized cells were not possible, due to their small dimensions. The amounts of polysaccharides are expressed in arbitrary PAS units (periodic acid Schiff) and are obtained by multiplying the extinctions by the square of the radius of the sphere of the acrosome.

The third and fifth lobes of the testis, which contain the large sized cells, show the same picture in regard to size, as well as the PAS color of the dictyosomes and their behavior in the formation of the acrosome. The lobes carrying the normal and small sized cells show a markedly smaller amount of acrosomal material, though essentially the same steps of development can be observed.

The formation of the acrosomal material from the dictyosomes in the large sized cells, as seen in slides treated with PAS and counterstained with methyl-green may be outlined as follows:

(*a*) In the confused stage of the first spermatocytes, the dictyosomes tend to form larger aggregates in the vicinity of the nuclear membrane. This dictyosomal material shows the characteristic red stain resulting from the PAS treatment, a stain not shown by the nucleus, nucleolus and the cytoplasm.

(*b*) In the course of the two spermatocyte divisions, the dictyosomal material is distributed approximately equally to the resulting spermatids, and is present in the latter as a granular mass. This constitutes the so-

called acroblast. The large Nebenkern does not stain after PAS at this or any later stage.

(*c*) The acroblast gives rise to the acrosome which at first is applied to one side of the spherical nucleus as a round cap, staining an intense red after PAS treatment.

(*d*) In the succeeding stage, this cap appears to become more liquid and extends over half or more of the still spherical nucleus.

(*e*) When the nucleus elongates, the acrosomal material elongates simultaneously. But the long, pointed acrosome of the finished sperm is not molded solely by the lengthening of the sperm nucleus, since it extends far beyond the anterior tip of the latter.

While all the earlier stages (*a–d*) show the characteristic methyl-green staining of the DNA in the nucleus, no such color is discernible with certainty in the final stages. Apparently this is due to the fact that the disproportionately large amount of PAS positive material in the large cells completely covers the nucleus of the elongated phase. That DNA is present in such spermatids in a normal quantity has already been demonstrated by Schrader and Leuchtenberger.[20] Indeed, in the small and normal sized spermatids, in which the quantity of DNA is the same as in the large cells just described, there is no difficulty in observing the methyl-green stain of the DNA in the nucleus, for the relatively much smaller amount of acrosomal material does not obscure it.

TABLE 1

COMPARISON OF AMOUNTS OF POLYSACCHARIDES (PERIODIC ACID SCHIFF REACTION) IN THE ACROSOME OF LARGE AND NORMAL SIZED SPERMATIDS OF ARVELIUS ALBOPUNCTATUS, BY MICROSCOPIC PHOTOMETRIC MEASUREMENTS

			ACROSOME		POLYSACCHARIDES, MEAN AMOUNT IN ARBITRARY PAS UNITS PER ACROSOME
LOBE OF TESTIS	TYPE OF CELL	NUMBER MEASURED	MEAN DIAMETER IN MICRONS	MEAN EXTINCTION	
Third (large sized cells)	Early spermatid	10	4.75	1.0 ± 0.02	5.76 ± 0.16
Sixth (normal sized cells)	Early spermatid	10	0.76	1.0 ± 0.03	0.14 ± 0.04

From the cytological studies there seems to be no doubt that the PAS positive material of the acrosome in the spermatids and sperms is derived from the PAS positive material in the dictyosomes of the primary spermatocytes (confused stage). A similar "gradual transformation of the Golgi material of the young spermatid into the sperm cap and acrosome," also by the use of the Hotchkiss reaction, has been observed by Leblond[3] in the rat testis.

The striking increase of the amount of PAS positive material in the acrosome of the large sized cells as compared with the amount in the

normal sized cells is demonstrated in table 1. On the basis of the measurements tabulated in table 1, it appears that the acrosome of the large-sized spermatids contains considerably more polysaccharides (about 40 times more) than the acrosome of the normal sized spermatids in the same meiotic stage. This increase in carbohydrates in the large sized cells is in accordance with the previously reported increase in proteins and ribosenucleic acid of these cells[9] and supports the concept of Schrader and Leuchtenberger that in Arvelius the increase in volumes of the nucleus, nucleolus and cytoplasm in the large sized cells, as compared with those of the normal and small sized cells, represents a true growth.

TABLE 2

EFFECTS OF VARIOUS REAGENTS AND ENZYMES ON THE POLYSACCHARIDES (AS DEMONSTRATED BY THE PERIODIC ACID SCHIFF REACTION) OF THE DICTYOSOMAL AND ACROSOMAL MATERIAL OF THE GERM CELLS OF THE TESTIS OF ARVELIUS ALBOPUNCTATUS

REAGENT	CONCENTRATION	TIME OF EXPOSURE	TEMPERATURE	PAS REACTION
Acetic anhydride + Pyridine	13 cc. + 20 cc.	45 min.	Room	Negative
Acetic anhydride + Pyridine followed by KOH	13 cc. + 20 cc. / 0.1 *N*	45 min. / 45 min.	Room / Room	... / Positive
Methanol chloroform	1:1	24 hrs.	60°C.	Positive
Saliva	Conc.	30 min.	Room	Positive
Amylase (Fisher Scientific "Amylopsin"	1%	60 min.	37°C.	Positive
Diastase (Merck U. S. P. IX)	1%	60 min.	37°C.	Positive
Schering hyaluronidase bull testis A	4 T.R.U. per 1 cc.	24 hrs.	37°C.	Positive
Schering hyaluronidase bull testis B	3.3 T.R.U. per 1 cc.	24 hrs.	37°C.	Positive
Wyeth hyaluronidase bull testis	140 T.R.U. / 70 T.R.U. per 1 cc.	24 hrs.	37°C.	Positive
Clostridium welchii hyaluronidase	.....	24 hrs.	37°C.	Positive

Some further analysis as to the chemical nature of this differential growth was attempted and from the results presented in the first two rows of table 2 it is evident that the chemical groups of the acrosomal material reacting with the Schiff reagent after periodic acid oxidation are aldehydes derived from 1,2 glycol grouping of carbohydrates. Using the reversible acetylation technique in tissue sections, as described by McManus and Cason,[13] the acetylation of the 1,2 glycols by acetic anhydride prevents the

formation of aldehydes after periodic acid oxidation and thus gives a negative PAS reaction, as seen in the first row of table 2. The removal of the acetyl groups from the acetylated 1,2 glycols by 0.1 *N* KOH restores the 1,2 glycol linkage and thus allows the formation of aldehydes after periodic acid oxidation, which color with the Schiff reagent, as seen in row 2 of table 2. That the positive PAS reaction is actually due to 1,2 glycols of carbohydrates and not to similar groups of glycolipids is shown by the experiments recorded in the third row of table 2; here the method devised by Gersh[4] showed that an extraction of glycolipids with hot methanol chloroform resulted in no effect on the positive PAS reaction. Furthermore it is obvious from table 2 that the polysaccharide content of the dictyosomal and acrosomal material is not due to the presence of starch or glycogen, because pretreatment of the cells with amylase, diastase or saliva did not change the positive PAS reaction. Control slides containing glycogen in liver cells, fixed and treated in the same manner as the testis slides of Arvelius, showed a negative PAS reaction of the glycogen granules after diastase and saliva treatment. It is further evident that different types of hyaluronidases, even in concentrations as high as 140 Turbidity Units (T.R.U.) per 1 cc., which readily digested the hyaluronic acid of umbilical cord, did not affect the PAS reaction of the acrosomal carbohydrates. These results more or less exclude the presence of hyaluronic acid in the acrosome and dictyosomal material of the germ cells of the Arvelius testis; although the possibility must be admitted that, due to a species specificity, the bull testis and bacterial hyaluronidases might not act on insect hyaluronic acid—or the substrate after fixation with Carnoy might be present in a form in which the enzyme is unable to attack it. It may be of interest to note that the treatment of the Arvelius testis slides with the enzyme solution of *Clostridium welchii* abolished the methyl-green stainability of the desoxyribose-nucleic acid in the nuclei of the cells and thus indicated the presence of a desoxydepolymerase in this enzyme solution. The cytochemical detection of a desoxydepolymerase in bacterial filtrates of *Clostridium welchii* is in good agreement with the observation of Warrack and coworkers,[21] who have obtained independently the same results by chemical means (personal communication).

Since the presence of polysaccharides with 1,2 glycol grouping in the dictyosomal material and in the acrosome of the male germ cells cannot be explained by its content of starch, glycogen or hyaluronic acid, the question arises as to the possible chemical nature of this substance. It is known that male germ cells of all species examined contain an enzyme hyaluronidase, which dissolves the cementing material surrounding the female germ cells and thus makes fertilization possible.[3, 11, 17, 18] Exceptions are the male germ cells of reptiles and birds, in which hyaluronidase has not been found and where accordingly the ova are not surrounded by

follicle cell cumuli.[6, 7] Moreover, the acrosome of the sperm has already been regarded by early workers, such as Lillie[9] and Bowen,[1] as being of great importance for the fertilization process, especially in connection with the penetration of the sperm and the activation of the egg. Bowen,[1] who pointed out the close analogy between the formation of the acrosome and that of a "secretory granule," has suggested that in the case of the acrosome the Golgi apparatus may be a center for the formation of enzymes which may play a part in the activation of the egg. The existence of enzymes in the sperm and their possible importance for the process of fertilization has already been stressed by Lillie[9] and Loeb.[10] The presence and possible role of the enzyme hyaluronidase in the acrosome and its elaboration by the dictyosomal material of the spermatocytes therefore demands consideration. The studies of Riisfeldt[16] demonstrating that during rat spermatogenesis the hyaluronidase is first found in the spermatocytes may be a corollary to our findings of the appearance of the dictyosomal material in the primary spermatocytes and suggest a possible relationship between dictyosomal material and hyaluronidase. Whether the 1,2 glycol grouping of the polysaccharides in the dictyosomal material and the acrosome might be indicative of the presence of the enzyme hyaluronidase itself must await further chemical characterization of the enzyme Studies in our laboratory, in which bull testis hyaluronidase was tested in vitro for 1,2 glycol groups, gave a positive PAS reaction. Moreover, a series of preparations of this enzyme, containing respectively 220, 550, 900 and 1400 T.R.U. per mg., showed a corresponding increase in the intensity of the PAS reaction. Since, according to Hotchkiss, the amount of dye fixed is dependent upon the actual weight of glycol structure present, it seems that the more purified the enzyme preparation (for instance 1400 T.R.U. per mg. as compared with 220 T.R.U. per mg.), the more 1,2 glycol groups can be demonstrated by means of the PAS reaction. Whether the 1,2 glycol groups are actually a part of the chemical constitution of the enzyme hyaluronidase, or whether they happen to be an "impurity" which became more concentrated during the purification process of the enzyme, must await the testing of enzyme preparations with higher T.R.U. per mg., which are not yet available. The speculation that the enzyme hyaluronidase contains 1,2 glycol linkage and thus gives a positive PAS reaction if present in tissues, led us to investigate the snake sperm, in which, as previously mentioned, no hyaluronidase has been found, and to compare it with the bull sperm, which serves as a good source for the extraction of hyaluronidase. While both sperms show a distinct acrosome, the snake sperm showed only a very slight amount of PAS positive material at the extreme tip of the acrosome, in contrast to the bull sperm where the whole acrosome, which consists of a thin hull covering two-thirds of the sperm head, was stained by the PAS reaction. That the PAS positive reaction in

the acrosome of the bull sperm is not due to starch, glycogen or hyaluronic acid was shown by pretreating sections with amylase, diastase and bull testis hyaluronidases without any effect on the intensity of the PAS reaction.

Summary.—Evidence is presented that the acrosome of the sperm in Arvelius albopunctatus is derived from the dictyosomal material of the primary spermatocytes and that the dictyosomal material and the acrosome contain a polysaccharide with a 1,2 glycol grouping which is neither starch, glycogen nor hyaluronic acid. The amount of polysaccharides is approximately 40 times larger in the acrosome of the sperms derived from the large sized cells than in that derived from the normal sized cells. The possibility of the relationship between acrosome and the enzyme hyaluronidase is discussed.

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[22] We prefer to call the Golgi apparatus in the germ cells dictyosomal material since the dictyosomes have been seen frequently in living germ cells, while such structures as the Golgi network now appear to be fixation artifacts.

[23] We are indebted to Dr. J. Seifter of the Wyeth Institute of Applied Biochemistry for the generous supply of various samples of bull testis hyaluronidase; to the Department of Biochemistry, Schering Corporation, for bull testis hyaluronidase; and to Drs. Pillemer and Robbins of the Institute of Pathology, Western Reserve University, for a bacterial filtrate of *Clostridium welchii*.

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ON THE DERIVATION OF SPACE DENSITIES IN GLOBULAR CLUSTERS

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In studies of the distribution of stars in globular clusters, the observations give directly only the distribution of stars or integrated light as projected on a plane perpendicular to the line of sight. It remains to deduce the spatial distribution from this areal distribution. Early investigators of the structure of globular clusters gave their attention to this problem, and solutions were given by von Zeipel[1] and Plummer[2] for the case of a spherically symmetrical cluster.

Von Zeipel showed that the density (of stars or integrated light) in space at a distance ρ from the center of the cluster is given by

$$\varphi(\rho) = \frac{1}{\pi}\int_{\rho}^{R} \sqrt{r^2 - \rho^2}\,\frac{d}{dr}\left(\frac{1}{r}\frac{df}{dr}\right) dr, \qquad (1)$$

where $f(r)$ is the density in projection at a distance r from the projected center, and R is the radius of the cluster. (R may be infinite.) Plummer used a different approach, starting by dividing the cluster into a number of parallel strips of infinitesimal width dr. If $F(r)dr$ is the total number of stars (or the sum of integrated light) in a strip whose perpendicular distance from the projected center of the cluster is r, then the space density is given by

$$\varphi(r) = -\frac{1}{2\pi r}\frac{dF}{dr}. \qquad (2)$$

Plummer's formula is plainly the simpler of the two; and in the reduction of star counts, where $F(r)$ follows directly from the summation of the number of stars in successive reticle squares, it is to be preferred. In measures of integrated light intensity, however, the observed function is $f(r)$, and $F(r)$ can be derived only by numerical integration along the length of each strip. The reduction by this method is just as laborious as by direct application of von Zeipel's formula; in fact it is easy to show that the two procedures are equivalent. There seems in the case of integrated light intensities to be no way of getting around the labor of applying von Zeipel's formula.

Another drawback of von Zeipel's formula is the error introduced in carrying out the required differentiations and integration. The observational errors—or the errors of smoothing the raw data—are magnified at each successive step of the calculation; and the differentiation or quadrature

formulae have residual errors which are often considerable, because the observed points are not as closely spaced as the numerical analyst might desire.

It is possible, however, to improve the practical accuracy of von Zeipel's formula by eliminating one of the differentiations. Partial integration of the formula gives

$$\varphi(\rho) = -\frac{1}{\pi}\int_{\rho}^{R} \frac{1}{\sqrt{r^2-\rho^2}}\frac{df}{dr}\,dr, \tag{3}$$

the integrated term vanishing. One differentiation has been eliminated, but the integral in this form is not amenable to straightforward numerical quadrature because of the divergence of the integrand at the lower limit.[3] Divergent integrands pose no such problem in analytic quadrature, however (provided the integral itself does not diverge); so we may remove the present difficulty by absorbing the divergent part of the integrand into the analytic quadrature leading to a new formula for numerical quadrature. In other words, we will write the integral as

$$\int_{\rho}^{R} \frac{1}{\sqrt{r+\rho}}\frac{df}{dr}\frac{dr}{\sqrt{r-\rho}} \tag{4}$$

and derive a new type of quadrature formula. Given a set of values of a function y, the new formula will give

$$\int y\,(r)\,\frac{dr}{\sqrt{r-\rho}} \tag{5}$$

(instead of the conventional $\int y(r)dr$) in terms of values of y.

Let $y_0, y_1, y_2, \ldots$ be the values of y at a number of equally spaced points $r_0\,(=\rho), r_1, r_2, \ldots$. We define a variable u by

$$r = r_0 + uh, \tag{6}$$

where h is the spacing $r_n - r_{n-1}$. We need formulae only for the integral over the strip from r_0 to r_1, since elsewhere the conventional quadrature formulae are applicable. Substitution of u for r as variable of integration gives for the required integral

$$I = \int_{r_0}^{r_1} y\,\frac{dr}{\sqrt{r-r_0}} = \sqrt{h}\int_0^1 y\,\frac{du}{\sqrt{u}}. \tag{7}$$

We can now derive various quadrature formulae, depending on which points we choose to pass the approximating polynomial through.

Let us first approximate y by a straight line passing through the points (r_0, y_0) and (r_1, y_1). The Lagrange interpolation formula gives for the equation of the line

$$y = y_0 (1 - u) + y_1 u, \tag{8}$$

and the approximation to the integral becomes

$$I = \sqrt{h} \int_0^1 [y_0(1 - u) + y_1 u] \frac{du}{\sqrt{u}} = \frac{2}{3} \sqrt{h} (2y_0 + y_1). \tag{9}$$

If we pass a quadratic through the points (r_{-1}, y_{-1}), (r_0, y_0), and (r_1, y_1), we get

$$y = y_{-1} \frac{u(u - 1)}{2} - y_0(u + 1)(u - 1) + y_1 \frac{u(u + 1)}{2} \tag{10}$$

$$I = \frac{2}{15} \sqrt{h} (- y_{-1} + 12y_0 + 4y_1). \tag{11}$$

And for a quadratic passing through (r_0, y_0), (r_1, y_1), and (r_2, y_2),

$$y = y_0 \frac{(u - 1)(u - 2)}{2} - y_1 u(u - 2) + y_2 \frac{u(u - 1)}{2} \tag{12}$$

$$I = \frac{2}{15} \sqrt{h} (9y_0 + 7y_1 - y_2). \tag{13}$$

Formulae giving a higher order of approximation may be derived in a similar manner. Note that (13) is generally less accurate than (11) and is to be used only where y_{-1} is unknown.

Von Zeipel's formula may thus be integrated numerically in the form (4). In the strip bounded by $r = \rho$ we use formula (9), (11), or (13), taking the function y as

$$y = \frac{1}{\sqrt{r + \rho}} \frac{df}{dr}. \tag{14}$$

Elsewhere we apply the usual quadrature formulae to (3). This method of deriving the spatial density distribution in a spherical star cluster is quicker and more accurate than the conventional application of von Zeipel's formula.

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[a] Society of Fellows, Harvard University.

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[2] *Monthly Notices R. A. S.*, **71**, 460 (1911).

[3] Von Zeipel gives (3) but passes over it as unsuitable for numerical evaluation.

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